Changes in innate immune function predict post-operative systemic inflammatory response syndrome (SIRS) following major Hepatico-pancreatico-biliary (HPB) surgery

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Submitted in fulfilment of the requirements of the degree of doctorate of medicine (MD(res))

Statement of originality

I, Rajiv Lahiri, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged.

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Abstract

Introduction

Patients undergoing major surgery are at risk of life-threatening complications including systemic inflammatory response syndrome (SIRS) and sepsis. Early identification of patients at risk of SIRS would allow tailored post-operative care and improve survival. Elevated serum IL-6 levels have been shown patients with poor post-operative outcomes, but the mechanisms underlying this response are unknown. We studied these mechanisms in patients undergoing major surgery to identify early biomarkers of altered inflammatory responses and poor clinical outcomes.

Methods

Serial blood samples were taken from consenting, adult patients undergoing major hepatic or pancreatic surgery pre-operatively and on days one and two post-operatively. Patients with inflammatory co-morbidities, pre-operative sepsis and those taking anti-inflammatory medications were excluded. Peripheral blood mononuclear cells (PBMCs) were isolated, stimulated for 24 hours with lipopolysaccharide (LPS) or flagellin and cytokine production was quantified by ELISA. PBMC surface expression of CD14, CD16, TLR4 and TLR5 was assessed by flow cytometry. Transcription factor phosphorylation was evaluated using Phosflow. SIRS was defined by internationally agreed consensus criteria.

Results

Serum concentrations of IL-6 on postoperative Day 2 were significantly increased in 12 patients who developed SIRS (median postoperative day 6) compared with 27 patients who did not. PBMCs from SIRS patients following surgery (before clinical signs of SIRS) displayed significantly greater TLR4 and TLR5 expression and produced significantly more IL-6 in response to LPS and flagellin. Consistent with these data, TLR-driven phosphorylation of NF-

κB was increased post-operatively, and interferon alpha-mediated STAT1 phosphorylation was higher pre-operatively in SIRS patients. Differences in TLR4 and TLR5 expression were greatest in the $CD14^{++}CD16^{+}$ 'intermediate' monocyte population. Intermediate monocyte TLR4 and TLR5 expression post-operatively predicted SIRS development with an accuracy of 0.89 - 1.0 (calculated areas under the receiver operator curves).

Conclusion

Markers of innate immune dysfunction can be used 5 days before the onset of clinical signs to identify patients at risk of SIRS.

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1. Changes in innate immune function predict post-operative sepsis following major HPB surgery

1.1 Overview

Post-operative sepsis remains a significant cause of morbidity and mortality following major abdominal surgery. This is especially true in hepatico-pancreatico-biliary (HPB) surgery, where patients undergoing major pancreatic and hepatic resections are susceptible to post-operative complications due to the technically demanding nature of the surgery. A large proportion of these complications relate to systemic inflammatory activity (largely related to bacterial infection). The innate immune system is known to play a role in the development of the systemic inflammatory response syndrome (SIRS) and sepsis following surgery. Key initiators of the innate inflammatory cascade are pathogen-sensing toll-like receptors (TLRs). Following binding of a specific ligand, innate inflammatory signalling pathways are activated and lead to the phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB subsequently translocates to the nucleus and binds to response elements, leading to pro-inflammatory cytokine production (e.g. IL-6 and TNF-α). Following surgery, dysregulated pro-inflammatory cytokine release is believed to be associated with post-operative complications.

There are currently no predictive markers that can determine which patients are at risk of post-operative sepsis following major surgery. In the early post-operative period, identification of patients at high risk of developing sepsis would allow tailored post-operative management and the use of specific therapies prior to patients developing clinical signs of sepsis.

The aim of this thesis is to evaluate the innate immune response to major HPB surgery in an effort to understand the changes in signalling that are associated with systemic inflammation and to identify pre-operative or early post-operative markers that highlight patients at high risk of developing post-operative sepsis when recovering from major surgery. Specifically, changes in monocyte phenotype, monocyte TLR4 and TLR5 expression, transcription factor phosphorylation and cytokine production will be evaluated pre- and post-operatively in patients undergoing major HPB surgery. These measures will be used to attempt to identify patients that go on to develop post-operative sepsis prior to this being determined by clinical and objective measures routinely used in post-operative care.

1.2 Systemic Inflammatory Response Syndrome (SIRS)

The systemic inflammatory response syndrome (SIRS) is a pro-inflammatory state that affects the whole body. When caused by an infection, it is termed sepsis. Other well described causes of SIRS include; post-operative complications, pancreatitis, trauma and burns (1). SIRS is thought to be initiated by common inflammatory pathways and was described as a 3 stage process (2) shown below.;

- **Stage 1** – Injury initiates local inflammation caused by local cytokine production. This local reaction acts to recruit the reticular-endothelial system and promotes wound healing.
- **Stage 2** – Cytokines enter the systemic circulation which initiates recruitment of macrophages and platelets. The response remains well controlled with a reduction in pro-inflammatory mediator release and an attempt to return to homeostasis.
- **Stage 3** – If homeostasis is not reached, on-going pro-inflammatory cytokine production can lead to systemic damage, activation of humoral processes and eventual loss of circulatory integrity. This can lead to end organ dysfunction.

This three stage description of SIRS pathophysiology was postulated in a systematic review article written by one of the authors that defined the criteria for diagnosis of SIRS (see 1.2.1). Focus was placed upon TNF-α, IL-1 and IL-6 as these were deemed to be the most important cytokines in the evolution of SIRS. The role of other cytokines in the 3 stage process is not included, although it is acknowledged that other cytokines are likely to play a significant role in SIRS pathophysiology (2). In addition, only patients with burns, trauma and haemorrhage, and pancreatitis were included in the review.

Current understanding of the pathophysiology of SIRS places emphasis on the elaboration of inflammatory cytokines TNF, IL-1 and Il-6 although many other mediators are also likely to

be involved. IL-6 production has been shown to increase significantly in patients with SIRS secondary to severe burns in human (3) and animal (4) studies. Further, marked or prolonged elevation of IL-6 appears to predict a poor prognosis in burns patients (3) (5). Even minor trauma leads to a rise in IL-6 levels in animal studies (6) with marked elevation following blunt chest trauma in humans (7). In patients who develop post-operative sepsis following elective surgery, IL-6 production was significantly greater from post-operative days 1 – 5 (8) than in healthy controls. The same group also demonstrated that IL-6 concentration greater than 310pg/ml was highly sensitive (90%) at predicting patients who developed post-operative sepsis (9). IL-6 concentrations have also been found to be elevated in patients that develop SIRS following hepatectomy (10). High IL-6 concentrations have also been shown to predict poor outcomes in patients with acute pancreatitis (11). Decrease over time in IL-6 concentration appears to confer an improved prognosis (12). Taken together, this variety of inflammatory models in animal and human series demonstrates the role of IL-6 in SIRS and appears to implicate this cytokine in stage three of the proposed pathophysiology. It also appears that IL-6 could have a role as a biomarker of post-operative SIRS.

The role of TNF-α in burns-associated SIRS is contradictory - results in human studies differ markedly, with evidence of stable TNF-α production (13) and significantly increased TNF-α production (14) reported in the literature. TNF-α concentration is elevated following trauma with major blood loss. This also occurred, although not to the same magnitude, when haemorrhage was not due to trauma (e.g. elective major abdominal surgery) (6) (15). However, no change in TNF-α concentration between patients with and without post-operative SIRS following major abdominal surgery has also been described (16). In a human series of severe pancreatitis, TNF-α concentration was significantly lower in patients that died compared to those that survived (17). These recent findings corroborate older results (18) and perhaps indicate that although high concentrations of pro-inflammatory cytokines may indicate a severe disease process, a subsequent marked decrease may be a pre-terminal event. Although a well described pro-inflammatory cytokine, the role of TNF-α in post-operative SIRS remains uncertain.

In patients with post-operative SIRS, innate immune cells (e.g. monocytes, macrophages) initiate the primary inflammatory response (19). This has been shown in human series (20) which demonstrated an alteration in human leukocyte antigen (HLA)-DR expression on monocytes. However, the heterogeneity of surgical cases studied could affect post-operative findings. Increased HLA-DR expression on monocytes occurs in elderly patients undergoing major abdominal surgery (21). However, the effects of co-morbidities or polypharmacy that may be relevant in an older population were not discussed.

SIRS can be initiated through binding of Toll-like receptors (TLRs) to their ligands (21), the signalling elements of which are detailed in a later section (1.3). Large quantities of TNF-α and IL-1 are released systemically early following an inflammatory insult and play a role in the release of stress hormones (e.g. adrenaline, noradrenaline), fever (22) and IL-6. IL-6 contributes to the production of acute phase proteins, such as C-reactive protein (CRP) and procalcitonin (23) (24). As described above, increasing concentration of IL-6 correlates with worsening prognosis following major trauma and surgery (25). There is evidence that IL-6 also has a role in the anti-inflammatory response by stimulating the production of prostaglandin E2, which in turn induces IL-10 (26) in trauma patients. Subsequently, the complement cascade is activated. In SIRS, there is evidence that the alternate pathway is activated prior to the classical pathway (27). C3, a protein common to both pathways appears to have an essential role in controlling bacteraemia as shown in $C3^{-/-}$ mouse studies (28). Pro-inflammatory cytokines are also involved in the production of nitric oxide and leukotrienes (29). Leukotriene production in patients with established SIRS was shown to be elevated on pLT-EIA plates (30) and animal studies demonstrated that MK-886, a leukotriene biosynthesis inhibitor reduced acute lung injury following haemorrhagic shock by reducing pro-inflammatory cytokine production (31).

Together with the release of pro-inflammatory mediators, inflammatory triggers also drive the compensatory anti-inflammatory response syndrome (CARS) (32), which acts in response to SIRS and the sequential activation of both systems can predispose to sepsis. In patients undergoing major abdominal surgery for gastrointestinal malignancy, IL-1ra and IL-10 production was significantly greater in patients that developed post-operative sepsis (8). However, this was only tested on patient serum by ELISA and cellular function was not evaluated. Another study assessed the balance of pro versus anti-inflammatory systems by evaluating the expression of monocyte HLA-DR. Low expression of monocyte HLA-DR on flow cytometry was associated with significantly increased risk of post-operative SIRS or sepsis following paediatric cardiothoracic surgery (33). In murine models of sepsis following caecal ligation puncture, both pro and anti-inflammatory cytokines were significantly elevated in mice that died within 24 hours (34). A broad panel of cytokines was assessed in the paper. With the growing literature available on SIRS and CARS, it appears that complex interaction between pro and anti-inflammatory pathways may lead to the development of sepsis. Further investigation is required, especially evaluating the roles of these systems after major abdominal surgery.

1.2.1 Definition and incidence of SIRS

Although there are numerous possible causes, SIRS can be summarised as occurring when common inflammatory pathways are stimulated inappropriately. In 1992, at the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference, criteria were agreed which allowed the diagnosis of SIRS on simple clinical and haematological parameters (1) . This was done to define a clinical response to a nonspecific insult of either infectious or non-infectious origin. SIRS can be diagnosed when 2 or more of the following variables are present;

Findings	Value
Temperature	<36°C or >38°C
Heart rate	>90 beats per minute
Respiratory rate	>20 breaths per minute or PaCO2 < 4.3kPa
White cell count	$<4x10^9$/L or $>12x10^9$/L

Table 1.1. The internationally agreed criteria for diagnosing SIRS. Two or more variables must be present.

A large retrospective study of over 500,000 patients identified a 13-fold increase in mortality in patients with post-operative SIRS, indicating the importance of early identification and treatment of this syndrome (35). In this study the reported incidence of post-operative SIRS was 3.6% although it included a variety of low-risk surgical specialties including ear, nose and throat (ENT) which have a lower incidence of SIRS than abdominal surgery. In elective patients undergoing open abdominal aortic aneurysm (AAA) repair, 89% of patients developed post-operative SIRS with a subsequent overall mortality of 4.8% (36). However, complications following open AAA repair are known to be greater than in patients undergoing pancreatic surgery. A retrospective analysis of 2322 patients that underwent distal pancreatectomy showed that sepsis, septic shock and pneumonia occurred in 16.7% of patients (37). Unfortunately, this study did not look into the incidence of post-operative SIRS in these patients, and did not comment on whether the procedures were open or laparoscopic. There are no studies evaluating the incidence of SIRS following pancreaticoduodenectomy (PD) and major liver resection. In light of the evidence presented, it is likely that both PD and major liver resection have a significant incidence of post-operative SIRS, and early identification could improve outcomes. Given the significant morbidity and mortality associated with major HPB surgery (see 1.2), early post-operative identification of patients at risk of SIRS and sepsis may lead to further improvements in post-operative care.

The overall incidence of SIRS in medical disorders beyond surgery is unknown. This is due to the heterogeneity of conditions that can lead to the diagnosis. It was noted in a prospective study that 68% of medical patients admitted to a tertiary centre met the diagnostic criteria for SIRS (40). Of the patients admitted with SIRS, 26% went on to develop sepsis (41). Sex differences in mortality due to SIRS are unknown, although the mortality in severe sepsis in women is significantly lower than in age matched men (42). Outcomes from SIRS are thought to be worst at the extremes of age and in patients with significant co-morbidities.

1.2.2 Complications of SIRS

As highlighted above, post-operative SIRS significantly increases the risk of mortality compared to patients that do not develop SIRS (35). This effect is seen across a variety of surgical specialties (35). Overall mortality depends on the cause, but in a prospective cohort study of 437 consecutive patients, there was an almost 7 fold increased risk of 28 day mortality in hospitalised patients with SIRS compared to those without (43). In patients that meet the criteria for SIRS, increased IL-6 levels (>300pg/ml) were associated with increased risk of multi-organ failure (MODS) and death (44).

As described above, inability to reach homeostasis leads to systemic damage and end organ dysfunction (2). A prospective cohort study of patients admitted to the emergency department showed that mortality from SIRS and sepsis syndromes has been shown to increase with increasing severity (from SIRS (2.1%) to septic shock (28%)) (45). This highlights the fact that it is organ dysfunction that leads to mortality. The most common systems affected include; the cardiovascular system (leading to hypotension and reduced tissue perfusion), the kidneys (causing acute kidney injury and renal failure) and the lungs (acute lung injury, which can develop into acute respiratory distress syndrome). A prospective 17 year longitudinal study which included over 11,000 ITU patients concluded

that mortality in patients with MODS was 22% (46). At a single HPB centre, patients that required second laparotomy for complications following pancreatic surgery had a high incidence of severe sepsis (36%) and MODS (32%) with an associated mortality of 48% (47).

1.2.3 Sepsis syndromes

SIRS due to a likely or a confirmed infectious organism is called sepsis (1). It is a potentially life-threatening condition that requires early recognition and immediate treatment. Sepsis following major abdominal surgery is almost invariably due to bacterial organisms, although circulating damage associated molecular patterns (DAMPs) are likely to contribute to the systemic inflammation. Sepsis is the initial stage of a progressively worsening clinical syndrome. Severe sepsis is defined as sepsis with end-organ dysfunction (e.g. elevated lactate, reduced urine output or hypotension). Septic shock is defined as severe sepsis with persistent low blood pressure in spite of intravenous (IV) fluid resuscitation (1). The pathophysiology of septic shock is not fully understood but a large number of cellular and humoral mediators are involved. Following major abdominal surgery, the most common causes of bacterial septic shock include; *E. coli*, *Streptococcus faecalis*, *Pseudomonas* and *Enterobacter*. Septic shock was shown to have poor prognosis, with mortality rates of around 29% (48) in a large observational cohort study that included over 190,000 patients from 847 hospitals. In post-operative patients diagnosed with septic shock, treatment is aimed at meeting three goals:

1. Use supportive measures to correct hypoxia, hypotension and impaired oxygen delivery to tissues.
2. Identify the source of infection.
3. Maintain end-organ perfusion to reduce the possibility of developing MODS.

Following a landmark study in 2001 (49), treatment of patients with septic shock involves a process termed early goal directed therapy (EGDT). This involves early broad spectrum intravenous antibiotic therapy and a 3 step approach. Step 1 involves central venous catheter (CVC) insertion and boluses of IV fluids until the central venous pressure is between 8-12mmHg in an attempt to correct the circulating volume (15). Step 2 requires arterial cannulation for continuous blood pressure (BP) monitoring and vasopressor therapy (e.g. noradrenaline) titrated to a mean arterial pressure (MAP) of at least 65mmHg to ensure adequate organ perfusion (49). Step 3 aims to correct tissue oxygenation by maintaining central venous oxygen saturation ($ScvO_2$) over 70% (50). This is done by ensuring maximum oxygenation and haemoglobin levels by blood transfusion. These measures were shown to improve mortality from septic shock by 16% (49). The principles of EGDT have been adopted as key features of the updated Surviving Sepsis guidelines in 2008 (51). A multicentre randomised control trial (RCT) comparing EGDT to standard management of septic shock demonstrated a significantly improved 28 day and ITU mortality in the EGDT group (52). Another prospective study in a single emergency department compared pre-EGDT outcomes to EGDT outcomes and found a 9% absolute mortality reduction (53). The largest multicentre RCT (ARISE trial) is currently recruiting patients to evaluate whether EGDT reduces mortality in septic patients compared to current practice. The ARISE trial aims to recruit 1600 patients, thereby making it the largest RCT in the literature. We currently extrapolate to the management of post-operative sepsis from studies such as this. However, our understanding of the pathophysiology of post-operative sepsis, risk stratification and optimal management has not been studied to the same extent and warrants further investigation.

Sepsis and the subsequent syndromes are potential complications of all major abdominal surgery. This is most commonly due to infected intra-abdominal collections, anastomotic leak or a damaged abdominal viscus. A recent epidemiological study demonstrated that the incidence of post-operative sepsis and severe sepsis has risen significantly over the past two decades in both elective and emergency settings (54). This could be due to an increase in complex surgical procedures in elderly patients, who are at highest risk of developing post-operative sepsis syndromes. Although the overall rate of post-operative sepsis in this study

was 1.02%, the study was evaluating a variety of surgical procedures performed by different specialties. Evidence assessing post-operative complications in hepato-pancreatico-biliary (HPB) patients demonstrates significantly higher rates of over 30% (55). This is potentially due to the technically demanding nature of HPB surgery, and to patients who often present with locally advanced malignancy. In spite of an increase in incidence of post-operative sepsis, the mortality rate in emergency cases has fallen significantly (54). This improvement could be due in part to improved recognition of high risk patients and the instigation of protocols such as 'surviving sepsis'. Although there have been improvements in mortality rates following post-operative sepsis in the emergency setting, these improvements have not been seen in elective patients (54). Efforts to identify an early predictive marker of post-operative sepsis could lead to improvements in elective patients undergoing major abdominal surgery.

SIRS commonly precedes sepsis. They are thought to be similar pathophysiological processes with associated cytokine dysfunction. Sepsis is defined as SIRS caused by infection and as described above, is associated with a significantly worse clinical outcome. Early warning scores are used regularly in the NHS to monitor the observations of patients. These early warning scores are based upon the criteria for SIRS, and inform doctors of patients at particular risk of developing sepsis. The rationale for studying SIRS as an endpoint is that it is objectively measured, can be diagnosed before evidence of microbial infection is apparent and therefore identifies patients at risk earlier in the sepsis pathway. Therefore, identification of an early marker of impending SIRS allows the opportunity to intervene even earlier in the pathogenesis and therefore (theoretically) prevent serious events.

1.2.4 Biomarkers of SIRS and sepsis

Mortality rates have been shown to increase significantly when SIRS is due to infection (55). A large multicentre prospective cohort study of ward and intensive care patients concluded that the mortality rate increased from 7% in SIRS to 16% in sepsis (40). This study was

performed before the advent of EGDT, which may explain the higher mortality rates in this study. Given the worse prognosis in cases of post-operative sepsis, efforts have been made to identify a biomarker that differentiates infectious and non-infectious causes of SIRS.

Over 170 potential biomarkers of sepsis have been evaluated in the literature. These include cytokines (IL-1β, IL-2, IL-6, IL-8, TNF-α), receptors (TLR2 and 4, TREM-1, TNF-receptor), acute phase proteins (CRP, procalcitonin), cell markers (CD14, CD40, CD80), coagulation markers (APTT, anti-thrombin) and markers of organ dysfunction (atrial natriuretic peptide, troponin) (56). Identification of potential biomarkers of sepsis has included a variety of experimental models and a number have been evaluated in clinical studies. The results of some of the clinical studies of these potential biomarkers are detailed below.

There is now evidence that procalcitonin (PCT) is more sensitive than CRP in identifying an infective cause of SIRS (57) (58) (59). A small cohort study of patients on ICU showed that PCT was more sensitive (85%) and specific (91%) at differentiating non-infective SIRS from sepsis than CRP, IL-2, IL-6, IL-8 and TNF-α (60). However, this was in a heterogenous population of critically ill patients on ICU. Another small prospective observational study indicated that in patients with established septic shock, PCT clearance was significantly greater in survivors compared to non-survivors (61). PCT has been evaluated at the onset of SIRS or sepsis following major surgery (62) (63), but does not appear to have a role in predicting which patients will develop post-operative SIRS or sepsis.

Other potential markers have been evaluated. In patients undergoing oesophagectomy, serial post-operative measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), PCT and IL-6 were able to distinguish between post-operative SIRS and sepsis (64), albeit not until post-operative day 3. Identifying patients with SIRS prior to the development of sepsis will likely lead to improved clinical outcomes. A small cohort study of 16 patients admitted to the ICU with multiple trauma injuries identified IL-1α, IP-10, sTNF-R2 and sFas as possible predictors of the progression of sepsis to septic shock (65).

This was performed as part of a Luminex panel assaying multiple cytokines and inflammatory proteins. Combined measurement of MMP-3, -10, IL-1α, IP-10, sIL-2R, sFas, sTNF-R1, sRAGE, GM-CSF, IL-1β and Eotaxin appeared sensitive (79%) and specific (86%) (65). However, measuring such a multitude of endogenous factors may prove unwieldy in clinical practice. A retrospective, case-control study evaluating changes in peri-operative gene expression concluded that combination of TNF-α, IL-1β and CD3D had a sensitivity of 90% and specificity of 85% of identifying patients that will develop sepsis 5 days prior to clinical diagnosis (65). The negative predictive value was 98.1%.

It was shown that early post-operative up-regulation of TLR2 and TLR4 receptors occurred in liver transplant patients that develop SIRS compared to those that did not (15). There is also evidence for the role of TLR2 and TLR4 in post-operative sepsis in mouse models. In mice with sepsis induced by caecal pole ligation and puncture, TLR2 and TLR4 gene expression and protein concentrations were significantly increased when compared to mice that underwent sham laparotomy(66). TLR2 and TLR4 gene expression was elevated in liver and lung tissue in mice with sepsis, however, tissue biopsy in septic patients is not feasible. Another study demonstrated that following liver resection, IL-6 and IL-8 levels are elevated immediately in patients that are at high risk of developing post-operative complications (67). However, IL-6 levels were significantly raised by duration of operation, which may confound these results. These findings were corroborated in another study investigating elective HPB surgery. In 56 patients undergoing major HPB resections, 30 patients developed post-operative infections. In these patients, IL-6 and IL-10 levels were elevated early in the post-operative period (68).

Currently, no single biomarker can predict the course of patients with sepsis with the degree of sensitivity and specificity to be employed in clinical practice. The use of several biomarkers may increase sensitivity, specificity and positive predictive value to allow routine use in clinical practice, but the incorporation of multiple markers may increase the processing time of the assay and would lead to considerable expense. Currently, no

individual biomarker has been validated in an independent cohort and is used in clinical practice.

Identification of an up-regulated receptor, subsequent differences in protein concentration in the signalling cascade and altered cytokine production may identify patients at risk of post-operative sepsis earlier than relying upon a single biomarker. In addition, receptors or phosphorylated signalling proteins could be targeted for inhibition as a potential therapeutic treatment. The discovery of early post-operative biomarkers that can accurately predict post-operative sepsis following major surgery might allow tailored post-operative management and early instigation of appropriate therapy to minimise the deleterious sequelae of sepsis.

1.3 Hepatico-pancreatico-biliary (HPB) surgery

HPB surgeons are responsible for the management of liver, bile duct and pancreatic resections, which are most commonly performed as part of the treatment for malignancies. Due to the vascular supply and anatomical location of these organs, the surgical approach is often complex with significant associated morbidity and mortality (69). The Cancer Outcome Guidelines (COG) were introduced in 1999 and implemented from 2003 (70) leading to centralisation of cancer services. This was done in an effort to improve outcomes following major cancer resections by ensuring that all HPB cancer cases were performed by experienced HPB surgeons in high volume centres (71) (72). Tertiary HPB centres in the UK should serve a population of at least 2 million. All possible cases of liver, bile duct and pancreatic malignancy are discussed at specialist HPB multi-disciplinary team (MDT) meetings.

The advent of centralisation has led to a significant reduction in morbidity and mortality (70). Improved post-operative care has also seen a reduction in length of hospital stay (69). However, post-operative morbidity and mortality rates remain high following major HPB resection due to the complex nature of surgery. The morbidity and mortality of each major resection will be discussed later in this chapter.

In accordance with changing surgical technologies, laparoscopy and, more recently, robotic surgery has been used in major HPB resections. Evidence supporting the use of these techniques over the traditional open surgical approach is currently weak, and is generally limited to case series (73) (74). Although laparoscopic and robotic approaches are technically feasible and should yield reduced blood loss and post-operative stay, a randomised controlled trial (RCT) comparing the different surgical approaches is required to eliminate bias and patient selection. There is significantly greater evidence for the use of laparoscopic distal pancreatectomy (75) (76) (77) and this has become the procedure of choice for tumours of the body and tail of the pancreas in certain UK HPB centres.

1.2.1 Pancreaticoduodenectomy (PD)

The pancreas is a retroperitoneal organ that is approximately 15cm in length (78). It is separated into three portions; the head, the body and the tail. The pancreas is intimately related to the duodenum medially, the posterior aspect of the stomach anteriorly, the spleen laterally and posteriorly the inferior vena cava, the right renal vessels, the left renal vein, the superior mesenteric vessels and the splenic vessels. The location and large vascular structures surrounding the pancreas are partially responsible for the complexity of pancreatic operations.

The most common major operations performed on the pancreas are; PD and distal pancreatectomy (DP). The indications for PD (Table 1.2) and DP (Table 1.4) are shown below.

Benign	Malignant
Benign ampullary neoplasm	Pancreatic ductal adenocarcinoma
Duodenal adenoma	Intra-ductal papillary mucinous neoplasm (IPMN)
Severe pancreatico-duodenal trauma	Ampullary carcinoma
Sphincter of Oddi dysfunction (all other treatment options unsuccessful)	Distal common bile duct (CBD) cholangiocarcinoma
Chronic pancreatitis	Duodenal carcinoma

Table 1.2. The indications for Pancreaticoduodenectomy

PD is a complex major procedure that takes 4-8 hours to complete. It was first described in 1935 by Allen Whipple and has now evolved into two variants; classical PD and pylorus preserving PD (PPPD). No significant differences in morbidity, mortality or long-term survival have been detected between the two variants (79). Evidence for different rates of intra-operative bleeding and post-operative delayed gastric emptying remains equivocal in the currently literature.

Surgical resection involves removal of the duodenum, head of pancreas, distal common bile duct (CBD), the gallbladder and part of the proximal jejunum. The procedure involves cholecystectomy and portal dissection to identify the CBD and portal vein at the hepatic hilum. The gastroduodenal (GDA) artery is identified as a major branch of the common hepatic artery (CHA), is dissected free and transfixed. The lesser sac is entered after division of the gastrocolic ligament and the duodenum mobilised (Kocher's manoeuvre) until the inferior vena cava (IVC) is visible and the posterior aspect of the pancreatic head is palpable. Identification of the superior mesenteric vein (SMV) and artery (SMA) inferior to the pancreatic neck allows the pancreas to be lifted off the portal vein, facilitating division of the pancreas. The proximal jejunum is transected and mobilised to the ligament of Treitz. Finally, the uncinate process is dissected free from the SMV and SMA to allow specimen removal. Following removal of the specimen, the upper gastrointestinal tract must be reconstructed. Many different reconstruction options are used, a common example is shown in Figure 1.1.

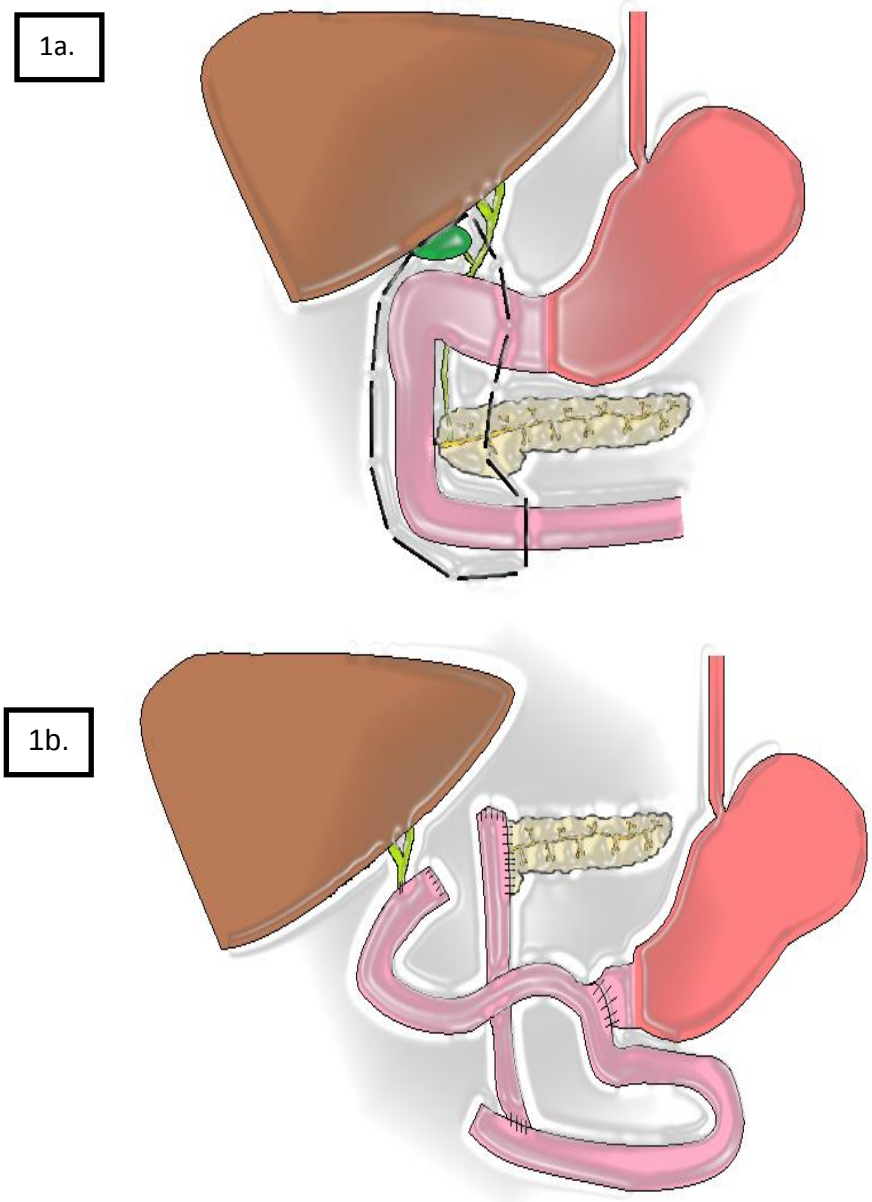


Figure 1.1. Figure 1a demonstrates the normal upper gastrointestinal anatomy. The dotted line indicates the organs that will be removed. Figure 1b shows a common reconstruction. An isolated loop of jejunum is anastomosed to the body of pancreas (pancreatico-jejunostomy). A limb of jejunum is anastomosed to the proximal CBD (choledocho-jejunostomy) and stomach (gastro-jejunostomy). The isolated loop of jejunum is anastomosed to the longer limb (jejuno-jejunostomy) to complete the reconstruction.

Other reconstructive options include pancreatico-gastrostomy. A number of studies have demonstrated no significant difference in complications between the two techniques (80) (81).

1.2.2 Complications following PD

As outlined above, major pancreatic resection is complicated by significant morbidity and mortality. Following centralisation of pancreatic services, 30 day mortality rates at major centres around the world are below 4% for PD (69) (70) (82) (83). In the largest series by a single surgeon in the literature, the mortality rate over a 37 year period was 1%. There was a reduction in operative time, morbidity, mortality and length of hospital stay as operator experience increased (84). Other major centres in the USA (85), UK (70) and Asia (86) have published case series demonstrating mortality rates of between 2-3.5%. Mortality following PD is most commonly due to sepsis following anastomotic leak. Other causes directly related to surgery are haemorrhage due to GDA stump blow out and liver abscesses and subsequent intra-abdominal sepsis. This is more common in cases of portal vein resection. Chest sepsis remains an important cause of post-operative mortality, especially in patients with significant co-morbidities.

Morbidity rates remain significant following PD. Literature from tertiary HPB centres around the world indicate morbidity following PD between 30-45% (70) (84) (86). The most significant cause of morbidity following PD is pancreatic anastomotic leak. This is documented in 5-20% of cases (69) (70) (82) (84). Wide bore external drains are inserted intra-operatively and placed adjacent to the pancreatic anastomosis. This allows external drainage of any possible pancreatic leak, with the aim of reducing the possibility of infected intra-abdominal collection. A study showed no difference in pancreatic leak rate between pancreatico-jejunostomy and pancreatico-gastrostomy (81). Pancreatic leak or fistula is graded by severity. This is shown in Table 1.3.

Criteria	No fistula	Grade A fistula	Grade B fistula	Grade C fistula
Drain Amylase level	<3 times the serum amylase	>3 times the serum amylase	>3 times the serum amylase	>3 times the serum amylase
Clinical conditions	Clinically well	Clinically well	Indeterminate	Clinically ill
Specific treatment	No	No	Yes/No	Yes
US/CT findings	Negative	Negative	Negative/positive	Positive
Persistent fistula drainage (>3 weeks)	No	No	Likely	Yes
Signs of infection	No	No	Yes	Yes
Readmission	No	No	No/Yes	Yes
Sepsis	No	No	No	Yes
Reoperation	No	No	Possible	Yes
Death related to fistula	No	No	No	Yes

Table 1.3. Grading of pancreatic fistula (adapted from (87)).

The majority of pancreatic leaks are grade A fistulae, which can be managed conservatively. In published case series from major HPB centres, not all groups define their pancreatic leak rate by the grading criteria in Table 1.3. Therefore, some centres document low overall pancreatic leak rates but a high incidence of re-operation, indicating that grade A and B fistula may not be included in their results. Other causes of morbidity due to sepsis following PD include; GI or biliary anastomotic leak, chest sepsis and wound sepsis.

The most common non-septic complications following PD is delayed gastric emptying (DGE). This can occur following stomach mobilisation and leads to prolonged NG aspirates and a delay in oral enteral intake. It can be managed by nasogastric (NG) aspiration and jejunal feeding or total parenteral nutrition (TPN). Complications can arise from the GDA stump, most commonly pseudoaneurysm, GDA-enteric fistula or GDA stump blowout with massive haemorrhage. GDA stump blowout can occur due to the local inflammation triggered by a pancreatic leak. Complications involving the GDA stump can generally be managed successfully by angiography and stenting. Other post-operative complications following PD include prolonged ileus and pancreatic insufficiency. Pancreatic insufficiency can affect both the endocrine and exocrine function of the gland.

Given that PD is a technically demanding procedure and there are numerous anastomoses required for reconstruction, including one involving the notoriously soft pancreas; it is perhaps unsurprising that there is a high incidence of post-operative septic complications. This has been described by numerous HPB units worldwide in the literature (70) (84) (86). Incisions in the upper abdomen are also more painful for patients. This leads to reduced mobilisation and shallow breathing, which can contribute to the development of chest sepsis.

1.3.3 Distal pancreatectomy (DP)

DP is performed for pathology limited to the body or tail of the pancreas. Technically, it is less complex than PD with lower associated morbidity and mortality (87). The indications for DP are shown in Table 1.4. Rates of resection for malignant lesions in the body or tail of pancreas are lower than for masses in the head of the pancreas as lesions in the body or tail present later due to the lack of CBD obstruction.

Benign	Malignant
Chronic pancreatitis	Pancreatic ductal adenocarcinoma
Benign cystadenoma	Cystadenocarcinoma
Mucinous cystic neoplasm	Neuroendocrine malignancy

Table 1.4. The indications for Distal Pancreatectomy

Distal pancreatectomy involves the removal of the body and tail of the pancreas. Due to the close proximity of the splenic hilum to the pancreatic tail and to ensure adequate oncological clearance, splenectomy is performed concomitantly for malignant lesions. The operation involves division of the gastrocolic ligament and into the lesser sac to visualise and mobilise the pancreatic body and tail. The greater curvature of the stomach is mobilised and the short gastric vessels ligated. The splenic artery is ligated prior to the splenic vein to

prevent engorgement of the spleen. Following transection of the pancreas, the specimen is removed and the pancreatic remnant is closed. It has been demonstrated that identification and suture ligation of the remnant pancreatic duct results in a significantly lower pancreatic fistula rate (87). In addition, a large international, multi-centre randomised control trial including 450 patients demonstrated no significant difference in pancreatic fistula rate following stapled or hand sewn closure of the remnant pancreas following distal pancreatectomy (83).

1.3.4 Complications following DP

DP is a complex major operation although 30 day mortality is less than 2% (87). A multi-site, international retrospective study analysed 302 consecutive DPs and found a morbidity rate of 35% and mortality of 2% (88). As in PD, mortality is most commonly due to sepsis following pancreatic leak. The grading for pancreatic leak severity following DP is shown in table 2. Morbidity following DP remains significant although it occurs less frequently than after PD. However, pancreatic leak remains a significant risk (around 30% in a recent RCT (83)) and it is common practice to insert a wide bore drain at the pancreatic suture line during surgery to externalise any possible pancreatic leak. Other significant complications include infected intra-abdominal collections, chest sepsis and pancreatic insufficiency. All patients that have concurrent splenectomy performed must be started on long-term prophylactic antibiotic therapy and require annual immunisations against *Streptococcus pneumonia*, *Haemophilus influenzae* type B and *Neisseria meningitides*. These measures are in place to prevent overwhelming post-splenectomy sepsis (OPSS).

1.3.5 Liver resection

The liver is the largest solid organ in the body. It sits in the right hypochondrium and is related to the anterior abdominal wall anteriorly, the diaphragm superiorly, the inferior vena cava and right kidney posteriorly and the gallbladder and transverse colon inferiorly. The liver has a rich blood supply from the common hepatic artery and primarily, the portal vein, which ensures that nutrients absorbed by the small and large bowel reach the liver for further metabolism. Claude Couinaud (a French anatomist and surgeon) anatomically defined the liver into 8 distinct segments based upon arterial supply and venous and biliary drainage (89) (Figure 1.2). The Couinaud classification is used by HPB surgeons to plan hemi-hepatectomy (removal of right or left liver lobe) or segmentectomy based upon the location of the lesion. The main indications for liver resection are shown in Table 1.5.

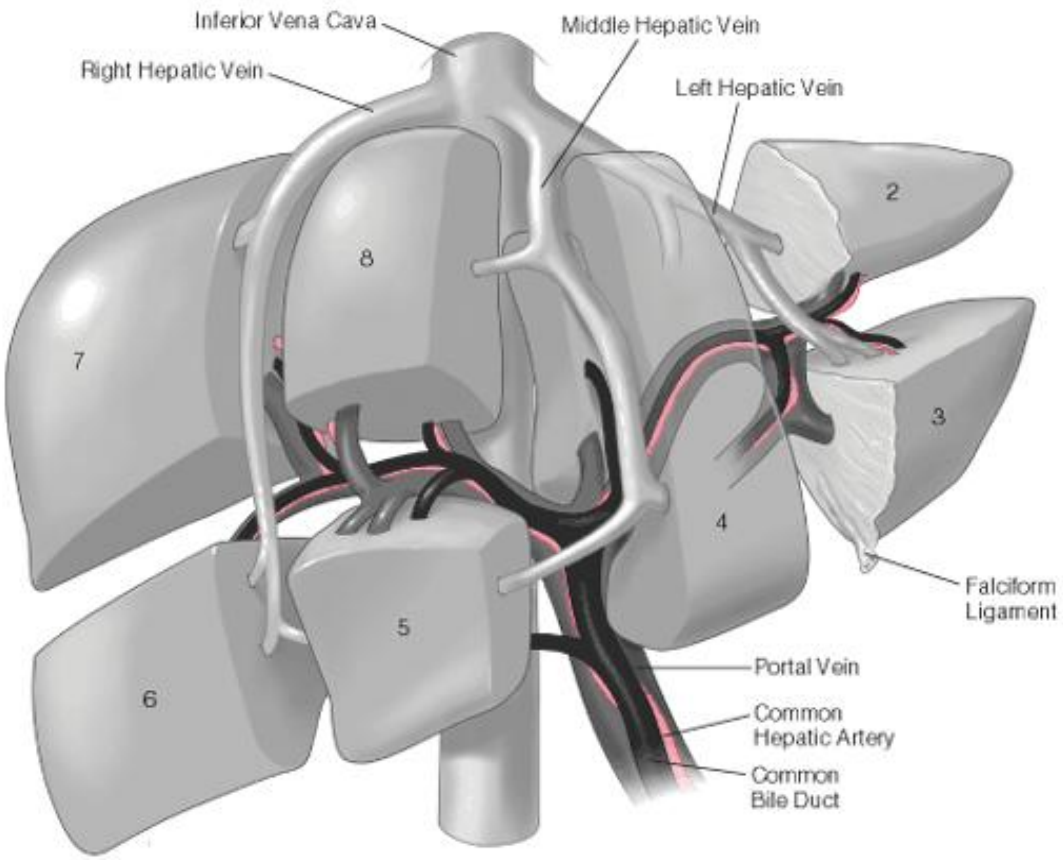


Figure 1.2. Couinaud's segmental anatomy of the liver. The left hepatic lobe consists of segments 1-4. Segments 5-8 comprise the right hepatic lobe. Segment 1 refers to the caudate lobe, which lies directly over the inferior vena cava (not shown). (taken from http://www.medscape.com/content/2003/00/44/98/449855/449855_fig.html on 13-03-2013).

Benign	Malignant
Hepatocellular adenoma	Colorectal metastases
Focal nodular hyperplasia (FNH)	Single hepatocellular carcinoma (HCC) less than 2cm in size
Hepatic haemangioma	Intra-hepatic cholangiocarcinoma

Table 1.5. The indications for liver resection

Anatomical liver resection refers to the removal of the left lobe (segments 2-4), the right lobe (segments 5-8) or specific segments of the liver (e.g. lateral segmentectomy – removal of segments 2 and 3). In a formal right or left hemi-hepatectomy, the middle hepatic vein should be preserved to ensure adequate venous drainage of the liver (89). More recently, efforts have been made to preserve liver parenchyma by performing non-anatomical resections, especially for peripheral lesions (e.g. peripheral lesion on segment 6). Although less liver parenchyma is removed in non-anatomical resections, blood loss can be higher, owing to the lack of vascular control during hepatic dissection (89).

To minimise intra-operative blood loss in major hepatic resections, vascular control is essential. Following mobilisation of the liver, the right, middle and left hepatic veins can be identified and controlled with vascular slings to prevent venous back bleeding. To control arterial and portal inflow to the liver, the hepatoduodenal ligament can be slung and controlled. This is known as the Pringle manoeuvre (90). Many surgeons use intermittent application of the Pringle manoeuvre during hepatic dissection. However, prolonged use should be avoided due to the risk of reperfusion injury.

The evidence for use of anatomical or non-anatomical resections remains weak. Retrospective analysis demonstrated no significant difference in patient survival between the groups for colorectal metastasis (91) or HCC (92). Randomised control trials (RCTs) are required to further inform HPB surgeons on the benefits of the two approaches.

1.3.6 Complications following liver resection

30 day mortality following major hepatic resection is below 4% in international tertiary HPB centres (93). Although mortality remains high compared to other elective gastrointestinal procedures, this is in part attributable to the co-morbidities many of these patients have as

a result of underlying liver disease. The majority of patients that undergo liver resection have had major colonic resections of primary colorectal cancers.

Bile leak is a significant cause of morbidity following liver resection. The largest retrospective case series in the literature identified a leak rate of 4%, with a high rate of subsequent mortality in these patients (94). Biliary leak can often be managed by percutaneous drainage, although it may require laparotomy if drainage does not improve symptoms. Hepatic failure can occur following extensive hepatic resection (right or left trisegmentectomies) and management is usually supportive in the intensive care setting as the remnant liver regenerates. If open major liver resection is performed, the upper abdominal incision can be susceptible to wound infection and patients are at risk of chest sepsis, due to pain on deep inspiration. This potential complication can be alleviated by a laparoscopic approach to major liver resection, which is gaining popularity. Retrospective studies indicate that there are fewer wound complications with a laparoscopic approach (95). However, to fully evaluate the benefits of minimally invasive techniques, a prospective RCT is required.

In summary, HPB surgery involves complex major resections of the pancreas and liver for malignancy. These operations are technically demanding and are performed at tertiary, high volume HPB centres by experienced surgeons. In spite of centralisation, post-operative morbidity remains high, mainly due to complications of the pancreatic anastomosis. Post-operative mortality has fallen significantly (70). In an attempt to identify surgical patients that are at high risk of developing post-operative sepsis, HPB patients provide ideal candidates for prospective investigation in the elective setting. In spite of good surgical technique, a significant number of patients will develop post-operative sepsis. As detailed in the previous chapter, innate immune responses play a significant role in the development of SIRS and sepsis. Evaluation of key proteins within the innate immune cascade like TLRs, NF-κB and pro-inflammatory cytokines may identify new early post-operative biomarkers of incipient sepsis which could lead to tailored management of patients before they develop overt sepsis.

1.4 Toll-like receptors (TLRs)

For many years, the innate immune system was thought to be a non-specific response to microbial stimuli leading to widespread inflammatory cytokine production. The discovery of Toll-like receptors (TLRs) demonstrated that innate immunity was more specific than previously understood and that as well as producing an immediate immune response (96) (97), the innate system has a role in mediating the adaptive immune response (98).

TLRs are transmembrane receptors that contain leucine-rich repeats (LRRs) at the N-terminus and a Toll–IL-1R (TIR) domain at the C-terminal cytoplasmic moiety (99). The Toll receptor was identified in Drosophila in 1995 (100) and the first TLR was discovered in humans two years later (97). Toll receptors are well conserved and are found in the nematode worm, *Caenorhabditis elegans*, to humans (101). The discovery of TLRs provided evidence that the innate immune system was more complex than previously thought and the presence of pattern-recognition receptors (PRRs) to detect specific parts of pathogens known as pathogen-associated molecular patterns (PAMPs) (102) indicated high levels of specificity. Since 1997, eleven functional TLRs have been identified in humans (Table 1.6) (100).

Toll-like receptor	Ligand	Location
TLR 1	Bacterial or spirochete lipoproteins	Ubiquitous
TLR 2	Bacterial or spirochete lipoproteins, Peptidoglycan	Myeloid, mast and natural killer cells
TLR 3	dsRNA	Intracellular within dendritic cells and natural killer cells
TLR 4	Lipopolysaccharide (LPS)	Monocytes, neutrophils, mast cells
TLR 5	Bacterial flagellin	Monocytes, dendritic cells, natural killer cells
TLR 6	Bacterial lipoproteins	Myeloid, mast, B cells
TLR 7	ssRNA	Intracellular within dendritic cells, B cells, eosinophils
TLR 8	ssRNA	Intracellular within myeloid, natural killer cells, T cells
TLR 9	CpG-DNA	Intracellular within dendritic cells, natural killer cells, B cells
TLR 10	Unknown	Dendritic cells, B cells
TLR 11	Uropathogenic bacteria	Dendritic cells, macrophages

Table 1.6. Principal ligands of human TLRs and their locations.

Broadly, the eleven described human TLRs can be split into two categories: the first group are expressed on the cell surface membrane and consist of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11. The PAMPs recognised by these receptors are lipoproteins and proteins, which are generally components of the microbial membrane. The remaining TLRs (TLR3, TLR7, TLR8 and TLR9) are located within intracellular vesicles and recognise microbial nucleic acids (102).

Binding of a PAMP to the appropriate TLR leads to activation of a signalling pathway. This leads to inflammatory cytokine and interferon (IFN) production (103). A more detailed description of the TLR signalling pathway is provided in section 1.4.3.

1.4.1 Toll-like receptor 4 (TLR4)

TLR4 was discovered in 1997 (97) and has since become the most widely studied TLR in the literature. The principal ligand for TLR4 is lipopolysaccharide (LPS) (104), a large molecule consisting of a lipid and polysaccharide that forms the major component of the cell wall of gram negative bacteria. LPS is endotoxin that induces a significant immune response and is engaged by the CD14-TLR4-MD2 complex on the cell surface. Lipopolysaccharide binding protein (LBP) also interacts with CD14 and presents LPS to the receptor complex (105). Five of the six lipid chains of LPS bind to the hydrophobic pocket of MD2 and the remaining lipid chain binds to TLR4 (106). Following LPS stimulation, the CD14-TLR4-MD-2 complex undergoes oligomerisation and activates downstream protein adaptors including; MyD88 (myeloid differentiation primary response gene 88), TIRAP (Toll-Interleukin-1 receptor domain-containing adaptor protein) and TRAM (TRIF-related adaptor molecule)

TLR4 signals through all three of these adaptor proteins. Other TLRs use a variety of adaptor proteins for downstream signalling (107). The majority of TLRs use the MyD88-dependent pathway, with the exception of TLR3 which signals through the MyD88-independent pathway. Although LPS is the principal ligand for TLR4, other agonists include taxol (108), morphine, oxycodone (109), carbamazepine (110) and endogenous molecules such as heat shock proteins (HSP 60 and HSP70) (108). However, very high levels of HSP60 or HSP70 are required to activate TLR4 signalling.

TLR4 and its co-receptors are highly expressed on monocytes and macrophages, indicative of the important role of these cells in the innate immune response. TLR4, MD2 and CD14 are

all expressed on neutrophils, but at low levels (111) and low levels of TLR4 are also expressed on B cells (112) in intestinal epithelium. As outlined above, TLR4 is unique in its ability to signal through both the MyD88-dependent and MyD88-independent pathways (107) (See 1.5). MyD88-dependent signalling leads to the rapid production of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-12 (113). One of the roles of rapid production of pro-inflammatory cytokines is neutrophil and macrophage recruitment and activation of the adaptive immune response.

The MyD88-independent pathway is activated through the adaptor protein TRIF (107). MyD88 knockout mice demonstrated delayed NF-κB and MAPK phosphorylation (114) and activation of the MyD88-independent pathway leads to the production of type I Interferons (IFNs) (115). Type I IFNs lead to the production of Interferon Stimulated Genes (ISGs) via the JAK and STAT proteins. ISGs have a role in anti-viral and antibacterial immune responses (115).

Following major abdominal surgery, the most common cause of post-operative intra-abdominal sepsis is anastomotic dehiscence (116) (117). This significantly increases morbidity and mortality following surgery (68). This is a particular risk factor following pancreatic surgery, due to the retroperitoneal location of the gland and its soft consistency. The organisms most commonly responsible for intra-abdominal sepsis following surgery are outlined in section 1.2.4. Intra-abdominal sepsis due to gram negative bacteria can lead to widespread TLR4 activation in response to LPS. Through the MyD88-dependent and independent pathways, TLR4 activation leads to widespread pro-inflammatory cytokine release which can lead on to SIRS and sepsis syndromes.

Changes in TLR4 expression following major abdominal surgery have been studied. TLR4 mRNA expression was significantly increased on post-operative day 3 in patients with post-operative infection following major HPB surgery in patient peripheral blood mononuclear cells (PBMCs) (68). However, these patients had established sepsis by day 3 and had

significantly more blood loss than patients without post-operative sepsis. Another group found a positive correlation between pre-operative TLR4 expression and gastrointestinal tumour stage (21). In this study, all recruited patients were diagnosed with either colorectal or gastric cancer. Following ileal pouch surgery for ulcerative colitis, patients were found to have significantly up-regulated TLR4 expression in active pouchitis compared to ileal biopsies from control patients (118). It appears that changes in TLR4 expression in the peri-operative period may have a number of roles in post-operative outcomes and warrants further investigation as a potential predictor of post-operative sepsis.

In addition to its role in inflammation and sepsis, TLR4 has been implicated in a number of non-infective disease processes. Animal models of cerulein-induced acute pancreatitis demonstrated over expression of TLR4 mRNA (119). It was also shown in mice that pre-treatment administration of a CD14 antibody prior to cerulein-induction and injection with LPS reduced the degree of pancreatic injury and pro-inflammatory cytokine production (120). TLR4 has also been implicated in the pathogenesis of atherosclerosis. TLR4 activation up-regulates intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule and MCP-1 in human coronary artery endothelial cells (121). This in turn increases TLR4 expression on macrophages allowing ingestion of oxidised lipids facilitating the transformation into foam cells (122) and contributing to the formation of atheromatous plaques. TLR4 is implicated in the pathogenesis of inflammatory bowel disease (IBD) as it has been shown that there is up-regulation of TLR4 on intestinal epithelial cells in IBD patients (123). TLR4 knockout mice are resistant to colitis-associated malignancy (124). In addition, over expression of TLR4 in surgical specimens of pancreatic ductal adenocarcinoma was associated with reduced long-term post-operative survival (125).

1.4.2 Toll-like receptor 5 (TLR5)

The only known ligand of TLR5 is flagellin (126), a globular protein that forms the principal part of bacterial flagellum. It is present in almost all flagellated bacteria; therefore, TLR5 is

at present, the only TLR known to recognise both gram-positive and gram-negative bacteria. Flagellin binds directly to the extracellular domain of TLR5 (127) and is highly specific. It can activate TLR5 signalling at extremely low concentrations. TLR5 was only thought to signal through the MyD88-dependent signalling cascade (126) (see 1.4.3) which leads to phosphorylation of NF-κB and production of pro-inflammatory cytokines (128). Recent evidence has highlighted the role of adaptor protein TRIF in TLR5 signalling in intestinal epithelial cells (129). In both TRIF knockout mice and TRIF-silenced NCM640 cells, flagellin stimulated primary intestinal epithelial cells produced significantly less NF-κB, JNK1/2 and ERK1/2 activation compared to controls (129). This indicates that TLR5 signalling is more complex than previously thought, with further efforts required to unveil other potential signalling pathways.

TLR5 is a cell surface receptor highly expressed on monocytes and macrophages. High levels are also found in the basolateral portion of intestinal epithelial cells suggesting that flagellin is only recognised when bacteria have crossed the epithelia (130). This indicates a possible role for TLR5 in gastrointestinal disorders. As flagellin recognises both gram-positive and gram negative bacteria, and TLR5 is highly expressed in the intestinal epithelium (131), it is feasible that TLR5 plays a significant role in post-operative sepsis following major abdominal (including HPB) surgery, although this has not been previously studied.

Elevated levels of TLR5 mRNA have been found in patients with ulcerative colitis (UC) compared to healthy controls on colonoscopic biopsies (132). The importance of TLR5 is gastrointestinal homeostasis was demonstrated in TLR5 knockout mice, who develop spontaneous colitis (133). TLR5 has been implicated in the development of gastrointestinal malignancy, as flagellin-induced ERK phosphorylation may contribute to the proliferation of gastric cancer epithelial cell line SNU638 (134). TLR5 appears to have a protective role against the development of metabolic syndrome as TLR5 knockout mice exhibit hyperphagia, hyperlipidaemia and insulin resistance (135). A common polymorphism ($TLR5_{392STOP}$) has also been implicated in disease. Flagellin binding does not initiate cell

signalling in the $TLR5_{392STOP}$ polymorphism. This leads to a susceptibility of pneumonia due to *Legionella pneumophila* (136).

1.4.3 TLR signalling pathways

Following PAMP binding and TLR stimulation, a rapid signalling cascade is activated which leads to the production of a variety of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α and interferons (IFNs)). Like many immunological signalling pathways, the appropriate functional response requires the presence of adaptor proteins, such as MyD88 (137). Our current understanding is that all TLRs (with the exception of TLR3) signal through MyD88 (138). TLR4 is also known to signal through TIR domain-containing adaptor inducing IFN-β (TRIF) (138) in the MyD88-independent pathway.

1.4.3.1 MyD88-dependent signalling pathway

The importance of MyD88-dependent signalling following TLR activation has been demonstrated by the attenuated inflammatory response in numerous knockout studies involving TLR2 (139), TLR4 (113), TLR5 (126) and TLR7 (140). In the MyD88-dependent signalling pathway (Figure 1.3), activation of IRAK-4 (IL-1 receptor associated kinase-4) is responsible for the recruitment and phosphorylation of IRAK-1 (22). The importance of IRAK-4 in the TLR signalling cascade has been shown in knockout studies where IRAK-$4^{-/-}$ mice show resistance to LPS-induced septic shock (141). The degradation of IRAK-1 activates TRAF 6 (tumour necrosis factor receptor-associated factor 6) by binding to the TRAF domain which, in turn, forms a complex with UBC13 (ubiquitin-conjugating enzyme 13) leading to the recruitment of TAK 1 (transforming growth factor-b-activated kinase 1) (142). IRAK-1 is degraded at the cell membrane and the TRAF6/TAK 1 complex moves into the cytoplasm. This complex activates the IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathways (22). The IKK family of proteins (IKKα, IKKβ, IKKγ) phosphorylate IκB (Inhibitor of κ

light chain gene enhance in B cells) which leads to degradation of IκB proteins. IκB proteins act as inhibitors of the NF-κB family by preventing dimerisation of NF-κB proteins (112).

Degradation of IκB is a critical step that permits translocation of the ubiquitous transcription factor NF-κB into the nucleus, and the expression of a large number of pro- and anti-inflammatory mediators such as cytokines and chemokines (22). Activated MAPK induces transcription factor AP-1, which also promotes the production of cytokines (143). Importantly, MyD88$^{-/-}$ macrophages still activate NF-κB and MAPK, indicating that MyD88-independent signalling to these transcription factors also exists.

Activation of these transcription factors are pivotal events in the initiation of an inflammatory response.

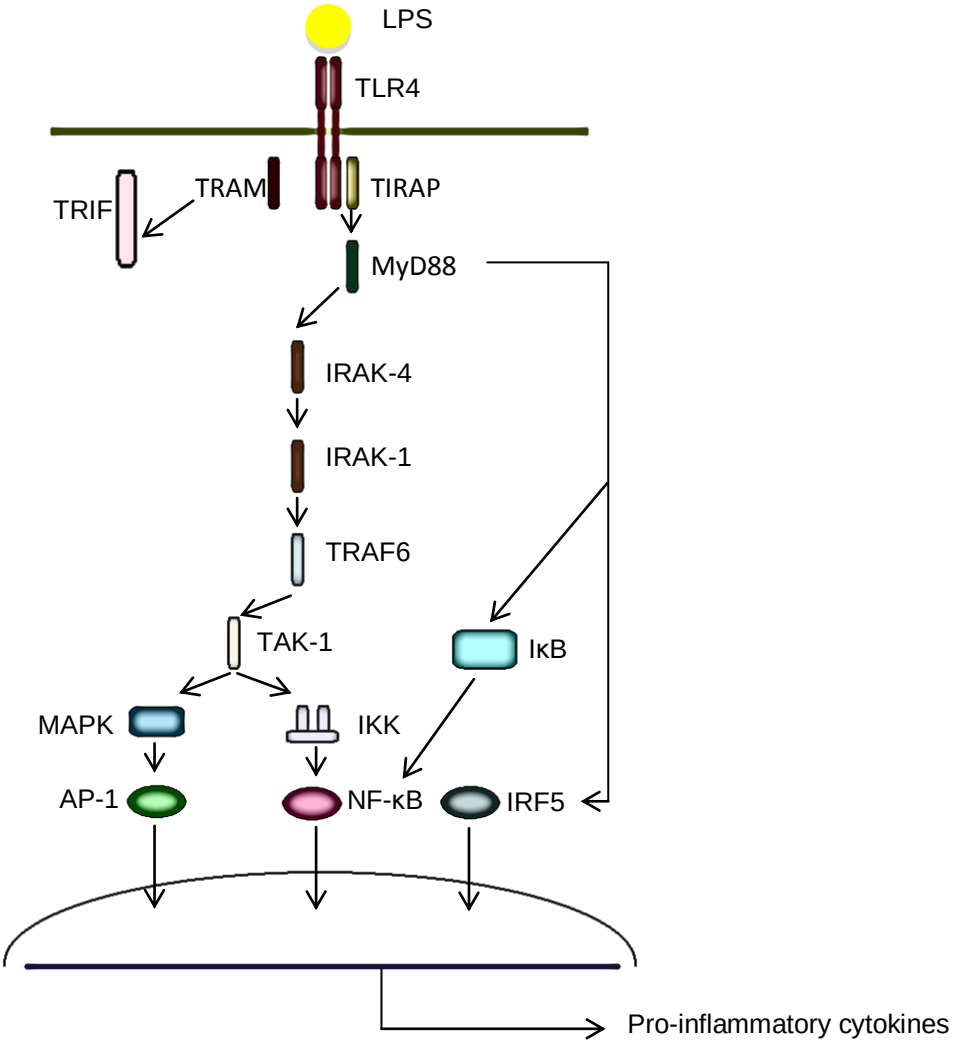


Figure 1.3. The MyD88-dependent signalling pathway following activation of TLR4 by LPS. Activation of IRAKs leads to induction of the transcription factors AP-1, NF-κB and IRF5. These transcription factors translocate to the nucleus and initiate pro-inflammatory cytokine release.

1.4.3.2 MyD88-independent signalling pathway

MyD88 knockout mice have reduced inflammatory cytokine production in response to most TLR ligands (113), with the exception of TLR4, which following LPS stimulation activates NF-κB and MAPK with delayed kinetics (113). The MyD88-independent pathway is mediated by the adaptor protein, TRIF (Figure 1.4). Deletion of TRIF leads to severely impaired IRF3, NF-ΚB and MAPK activation (144). The C terminus of TRIF interacts with RIP1 (receptor-interacting protein 1) to activate the IKK family of proteins and MAPK to induce NF-ΚB and AP-1 respectively (106). TRIF recruits TRAF3 which in turn associates with TANK (TRAF family member-associated NF-κB activator) and TBK1 (TANK binding kinase 1) to induce the transcription factor IRF3 (145). IRF3 and NF-κB initiate the transcription of target genes, including type I interferons (146). This in turn activates JAK/ STAT phosphorylation leading to interferon stimulated gene production (147). The importance of the MyD88-independent pathway is shown in TRAF 3-deficient cells, in which type I IFN induction is defective (148).

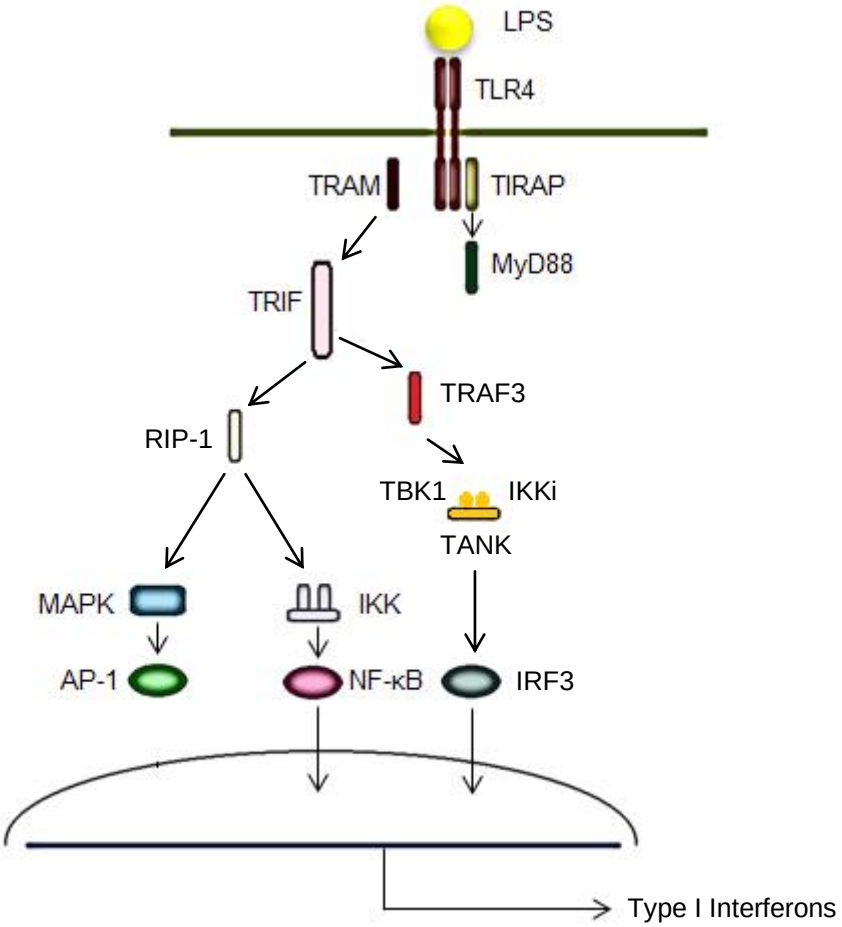


Figure 1.4. The MyD88-independent signalling pathway following activation of TLR4 by LPS. TRIF activation recruits RIP-1 and TRAF3 which leads to the induction of AP-1, NF-κB and IRF3. This cascade initiates the production of type I Interferons.

It is essential that effective inhibitory pathways are in place to prevent host damage through inflammation (22). Negative regulation of TLR signalling is complex and occurs at different points of the signalling cascade. Inhibitors on the cell surface are RP105 (radio-protective 105), SIGIRR (single immunoglobulin IL-1R-related molecule) and IL1Rl inhibit TLR signalling at the beginning of the signalling cascade (149). RP105 is specific for TLR4 and dendritic cells from RP105$^{-/-}$ mice produce large amounts of pro-inflammatory cytokines in response to LPS stimulation (150). SOCS1 (suppressor of cytokine signaling-1) and TRIAD3A (triad domain-containing protein 3 variant A) are E3 ubiquitin protein ligases (151). SOCS-1 inhibits the JAK–STAT signalling pathway and induces the ubiquitination of TIRAP. TRIAD3A can induce

the degradation of TLR4, TIRAP and TRIF, thereby inhibiting multiple TLRs (notably TLR4 and TLR9) (152). Further down the intracellular signalling pathway, IRAK-M prevents the IRAK family of proteins from disassociating from MyD88 thereby inhibiting signalling. This has been demonstrated in IRAK-M deficient macrophages, which have a higher inflammatory cytokine production in response to LPS (153). MyD88s is a splice variant of MyD88 which lacks a functional domain and therefore inhibits MyD88 signalling (154). TRAF1 and TRAF4 negatively regulate TLR4 signalling. TRAF1 is cleaved by caspases and then inhibits TRIF-induced NF-κB and IRF3 transcription factors (22). TRAF4 is thought to inhibit NF-κB induction by interacting with TRIF and TRAF6 (155). Another negative regulator is A20, a de-ubiqutinating enzyme. A20 inhibits NF-κB activation by removing ubiquitin moieties from TRAF6 (156). $A20^{-/-}$ macrophages produced more TNF-α, IL-6 and nitric oxide than wild type macrophages (154).

1.5 Preliminary work

Previous work in our laboratory had developed model in which to assess innate immune responses in patients' peripheral blood mononuclear cells (PBMCs) by determining the ex vivo production of TNF and IL-6 in CD14+ monocytes following stimulation with LPS or flagellin. Significant differences in inflammatory cytokine production between patients with non-alcoholic fatty liver disease (NAFLD), non-cirrhotic alcoholic liver disease (ALD) and patients with cirrhosis (Alazawi et al. Unpublished). Patients with compensated cirrhosis have a selective defect in flagellin-induced IL-6 production compared to patients with non-cirrhotic ALD or NAFLD ($p<0.01$). However, there was no difference in flagellin-stimulated TNF-α production or serum IL-6, TNF- α and RANTES concentration. This data indicated that there was selective impairment of TLR5-mediated IL-6 production in patients with compensated cirrhosis compared to non-cirrhotic patients. This model of innate immune activation is the basis of the assays I refined and developed in the course of the work presented in this thesis.

Preliminary data from the UK Genomic Advances in Sepsis (GAinS) study have implicated differences in ISG expression in patients who survived sepsis due to pneumonia compared to those who did not, strongly suggesting an innate immune influence on survival from system inflammation. Therefore I sought to evaluate peri-operative changes in innate immune function to determine if changes in TLR4 and TLR5 expression, transcription factor phosphorylation and cytokine production could identify patients at risk of developing post-operative SIRS.

1.6 Aims

A proportion of patients develop post-operative complications following major surgery. It is unclear why some patients develop complications and others do not but the magnitude of the host response to surgical trauma may be involved. My hypothesis is that patients who develop post operative complications have a more marked innate immune response and that pre or peri-operative identification of such patients may allow patient stratification into high and low risk groups, thereby facilitating more appropriate interventions. The overarching aim of this work was to examine innate immune dysfunction following major surgery in patients who did or did not develop complications. Specifically, we planned to evaluate peri-operative changes in monocyte TLR4 and TLR5 signalling following major HPB surgery and to determine whether changes could predict the onset of post-operative SIRS and sepsis. Three hypotheses have been evaluated;

1. IL-6, TNF-α and IL-10 are involved in post operative SIRS and their post operative production is significantly greater in patients that develop post-operative SIRS and sepsis compared to those that have an uneventful recovery.

2. Monocyte TLR4 and TLR5 expression is significantly greater in patients that develop post-operative sepsis compared to those that do not.

3. NF-κB phosphorylation is a key component of the inflammatory pathway and the level of activation differs significantly in patients that develop post-operative SIRS and sepsis compared to those that do not.

2. Methods

2.1 Patient selection

Patients undergoing complex hepato-pancreatico-biliary (HPB) surgery at Barts Health NHS Trust were recruited between August 2011 and May 2013. The Barts Health HPB Centre is a busy, tertiary referral centre that specialises in the management of complex diseases of the pancreas, liver and bile ducts. Demographic, clinical and pathological data were collected prospectively from review of patients' notes and the Electronic Patient Record (EPR) system.

2.1.1 Inclusion and exclusion criteria

Patients undergoing major hepatectomy, pancreatic resection and biliary reconstruction over the age of 18 years with capacity to consent were approached for inclusion in the study (Ethics approval was obtained from the East London Ethics Committee - REC number P/01/023). Patients were excluded if any of the following were present; active concurrent inflammatory disease (e.g. inflammatory bowel disease, rheumatoid arthritis), pre-operative sepsis and treatment with regular anti-inflammatory medications. Healthy volunteers were consented and recruited from the Blizard Institute.

2.2 Blood sampling

Blood sampling	Other equipment
2 heparinised vacutainers (BD Vacutainer Systems, Plymouth, UK)	Disposable tourniquet
1 serum-separating tubes (BD Vacutainer Systems, Plymouth, UK)	Non-sterile gloves
Vacutainer collection system	Alcohol steret wipe
	Cotton wool

Table 2.1. Equipment required for venous blood sampling.

25ml of blood was taken pre-operatively following consent. A further 25mls was taken on day one and day two post-operatively at clinically indicated times. Following major pancreatic, hepatic or biliary surgery, all patients were nursed on the high dependency unit (HDU) for 48 hours and all patients had a central venous catheter (CVC) inserted during the anaesthetic as per routine post-operative care. Both day one and two blood samples were taken using the CVC. 20mls of blood were collected from all patients in heparinised vacutainers (BD Vacutainer Systems, Plymouth, UK). 5mls of blood were collected in serum-separating tubes (BD Vacutainer Systems, Plymouth, UK) (Table 2.1). The samples were taken to the laboratory for serum and PBMC separation.

2.2.1 Serum separation

Serum separation	Other equipment
Serum-separating tubes (BD Vacutainer Systems, Plymouth, UK)	Centrifuge
	P1000 pipette
	P1000 pipette tips
	1.5ml eppendorf

Table 2.2. Equipment required for serum separation.

Following blood collection, serum-separating tubes were centrifuged at 2500rpm for 10 minutes. 1.5ml of serum was aspirated without disturbing the red cell pellet and immediately stored in labelled Eppendorfs at -80°C (Table 2.2).

2.3 Peripheral blood mononuclear cell (PBMC) separation

PBMC separation	Cryopreservation
Heparinised Vacutainer tubes (BD Vacutainer Systems)	1.8ml cryopreservation tubes
50ml Falcon tubes	10% DMSO Freezing mixture (5ml DMSO, 45mls foetal calf serum)
Ficoll-Paque™ Plus (GE Healthcare)	Mr Frosty® (isopropanol for refill)
Phosphate buffered saline (Invitrogen)	
Calibrated pipettes 10-1000µL	
Sterile pipette tips	
Sterile Pasteur pipettes	

Table 2.3. Equipment required for PBMC separation.

Phosphate buffered saline (PBS) (Invitrogen, New York, USA) was placed in a water bath for 10 minutes at 37°C. 20ml of blood from the heparinised vacutainer was then mixed with 20ml PBS in a 50ml Falcon tube. The resulting mixture was slowly layered onto 10ml Ficoll Paque (GE Healthcare, Little Chalfont, UK) and centrifuged at 2500rpm for 20 minutes with the brake off at room temperature. The resulting interface was aspirated with a Pasteur pipette into another 50mls Falcon tube and washed with 10ml PBS. The PBMCs were then centrifuged at 1700rpm for 5 minutes. The PBS was discarded and the cell pellet re-suspended in 10ml PBS and centrifuged again at 1700rpm for 5 minutes. The cell pellet was then re-suspended in 1ml of 10% DMSO freezing solution and placed in a Mr Frosty storage container with isopropanol for 24 hours at -80°C. Once frozen, the ampoule of PBMCs was then placed in a storage box at -80°C (Table 2.3).

2.4 Agonist stimulation

Thawing the cells	Agonist stimulation
Water bath at 37°C	LPS (stock solution 2mg/ml)
Complete medium (RPMI-1640, 50mls 10%FCS, 1% L-glutamine, penicillin/streptomycin)	Flagellin (stock solution 1mg/ml)
15ml Falcon tube	12-well tissue culture plate
Haemocytometer	
Microscope	

Table 2.4. Equipment required for agonist stimulation.

Complete medium (RPMI 1640 containing foetal calf serum (FCS), penicillin and streptomycin) was warmed in a water bath at 37°C for 15 minutes. Each ampoule of PBMCs was thawed and centrifuged at 1700rpm for 5 minutes. The resulting pellet was re-suspended in 1ml of complete medium and the cells counted using a haemocytometer. 2 x 10^6 PBMCs were then re-suspended in 1mls of complete medium and distributed into a labelled 12 well plate (1ml/well). The cells were then stimulated with 10µl (working concentration 2ng/ml) of lipopolysaccharide (LPS) and 2µl (working concentration 1mg/ml) of flagellin. LPS was used at a concentration of 2ng/ml and flagellin at 2µg/ml as previous experimental work from our group had confirmed these doses to produce optimum cytokine production in concentration gradient experiements. The plates were incubated at 37°C for 24 hours. Following incubation, the medium containing PBMCs was aspirated from each well. 1ml of complete medium was added to each well to free any cells adhered to the base of the tissue culture plate. The cells were centrifuged at 1700rpm for 5 minutes and the supernatants were removed. Supernatants were stored in Eppendorfs at -20°C until required for ELISA (Table 2.4). The PBMCs were then used for flow cytometry.

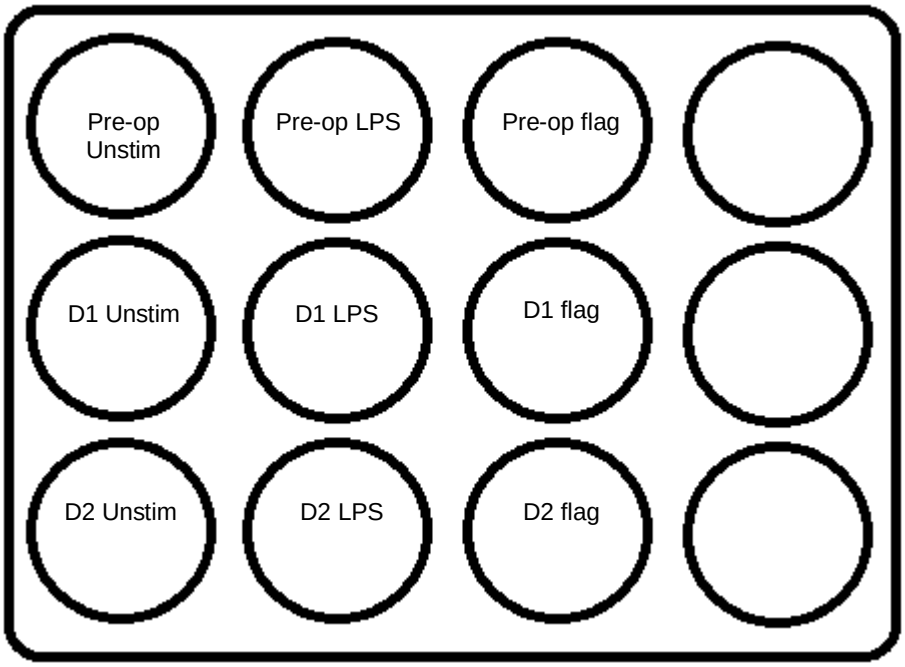


Figure 2.1. 12 well tissue culture plate with different experimental conditions. Unstim – unstimulated PBMCs pre-operative, day one and day two post-operative. LPS – LPS stimulated PBMCs pre-operative, day one and day two post-operative. Flag – flagellin stimulated PBMCs pre-operative, day one and day two post-operative.

2.5 Measurement of cytokines – ELISA

IL-6 ELISA (R&D Systems)	TNF-α ELISA (R&D Systems)	IL-10 ELISA (R&D systems)
Capture antibody. Stock solution -720µg/ml. Working concentration - 4µg/ml.	Capture antibody. Stock solution - 720µg/ml. Working concentration - 4µg/ml.	Capture antibody. Stock solution – 360µg/ml Working concentration - 2µg/ml.
Standard antibody. Stock solution - 340ng/ml. Working concentration - 1000pg/ml.	Standard antibody. Stock solution - 340ng/ml. Working concentration - 1000pg/ml.	Standard antibody. Stock solution – 95ng/ml Working concentration – 2000pg/ml.
Detection antibody. Stock solution - 45µg/ml. Working concentration - 250ng/ml.	Detection antibody. Stock solution - 45µg/ml. Working concentration - 250ng/ml.	Detection antibody. Stock solution – 27µg/ml. Working concentration -
Streptavidin Horseradish peroxidase (HRP)	Streptavidin Horseradish peroxidase (HRP)	Streptavidin Horseradish peroxidase (HRP)
PBS	PBS	PBS
Wash buffer (0.05% Tween®20 (Sigma-Aldrich) in PBS)	Wash buffer (0.05% Tween®20 (Sigma-Aldrich) in PBS)	Wash buffer (0.05% Tween®20 (Sigma-Aldrich) in PBS)
Reagent diluent (1% Bovine Serum Albumin (Sigma-Aldrich) in PBS)	Reagent diluent (1% Bovine Serum Albumin (Sigma-Aldrich) in PBS)	Reagent diluent (1% Bovine Serum Albumin (Sigma-Aldrich) in PBS)
Substrate solution (TMB (3, 3', 5, 5'-tetramethylbenzidine)	Substrate solution (TMB (3, 3', 5, 5'-tetramethylbenzidine)	Substrate solution (TMB (3, 3', 5, 5'-tetramethylbenzidine)
Stop solution (2N H_2SO_4)	Stop solution (2N H_2SO_4)	Stop solution (2N H_2SO_4)
96 well ELISA plate	96 well ELISA plate	96 well ELISA plate

Table 2.5. Equipment required for ELISA.

IL-6, TNF-α and IL-10 were measured using commercially available enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA). A 96 well plate was coated with capture antibody that was diluted in PBS to the working concentration. Each well was coated with 100µl of capture antibody. The plate was sealed and incubated overnight. The following day, the plate was washed with wash buffer four times and blotted dry with paper towels. 200µl of Reagent Diluent was added to each well to block the plate. This was left at room temperature for at least one hour. The wash step of the ELISA was repeated and a serial dilution of the standard antigen was made. 100µl of supernatant was added in duplicate and the plate incubated for two hours at room temperature. The wash step was repeated and 100µl of detection antibody (at working concentration) was added to each well. The plate was again incubated for two hours at room temperature. After repeating the

wash step, 100µl of the working concentration of streptavidin-HRP was added to each well. This was incubated for twenty minutes at room temperature out of direct light. The wash step was repeated and 100µl TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate solution was added to each well and the plate was incubated for a further twenty minutes out of direct light. The colour reaction was stopped with 50µl/well of 4N sulphuric acid. The plate was immediately read using a microplate reader set at 450nm with wavelength correction set at 570nm (Table 2.5).

2.6 Flow cytometry

Antibody	Fluorchrome	Isotype control
Human anti-CD14 (Biolegend, Cambridge, UK)	Pacific blue	Mouse IgG2a
Human anti-CD284 (TLR4) (Biolegend, Cambridge, UK)	Phycoerythrin (PE)	Mouse IgG2a
Mouse anti-TLR5 (Abcam, Cambridge, UK)	Fluorscein isothiocyanate (FITC)	Mouse IgG2a
Human anti-CD16 (Biolegend, Cambridge UK)	Alexafluor 647®	Mouse IgG1

Table 2.6. Antibodies and isotype controls used in monocyte analysis.

Solutions and reagents	Equipment
Complete medium (RPMI-1640, 50ml 10%FCS, 1% L-glutamine, penicillin/streptomycin)	12 well tissue culture plate
FACS buffer (500ml PBS, 10mls Foetal calf serum (FCS), 0.1g sodium azide, 0.18g EDTA)	FACS tubes
1% Paraformaldehyde (PFA)	Centrifuge
	P1000, P200 and P20 pipettes
	Pipette tips
	Canto II (BD Biosciences, Oxford, UK)

Table 2.7. Equipment required for flow cytometry analysis of TLR expression on monocytes.

2.6.1 Measurement of TLR4 and TLR5 cell surface expression on monocytes

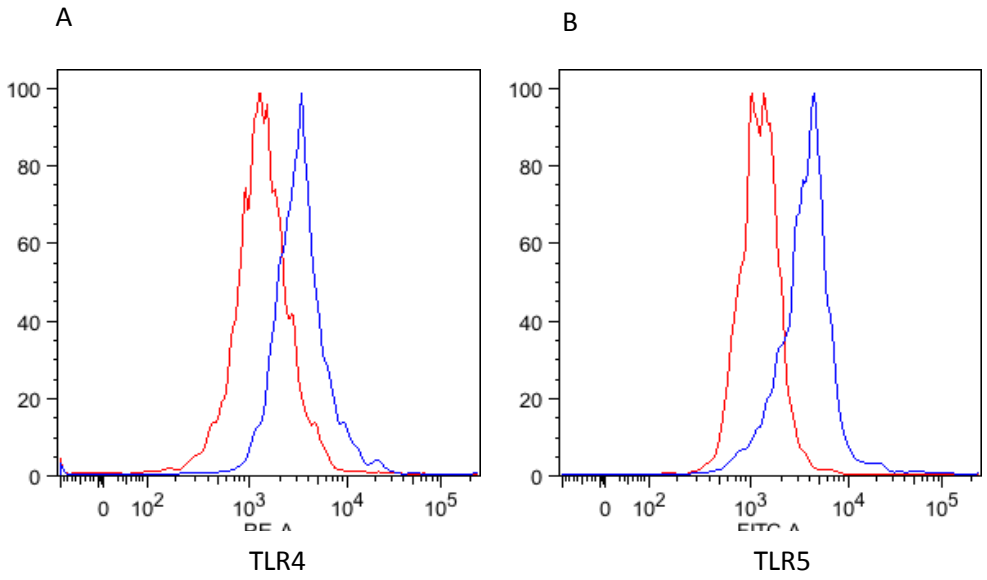
Following 24 hour stimulation, PBMCs were aspirated from each well into labelled FACS tube. Each well was washed with 1ml of complete medium to collect any cells that adhered to the base of the tissue culture well. The FACS tubes were centrifuged at 1700rpm for 5 minutes and the medium removed. The cells were re-suspended in 2ml of FACS buffer (500mls PBS, 10mls FCS, 0.1g sodium azide, 0.18g EDTA) and centrifuged at 1700rpm for 5 minutes. This process was repeated and the FACS buffer was removed from each tube.

CD14, CD16, TLR4 (Biolegend, Cambridge, UK) and TLR5 (Abcam, Cambridge, UK) antibodies were added to the PBMCs. The PBMCs were incubated at room temperature in the dark for 45 minutes. Following incubation, 2ml of FACS buffer was added to each tube and centrifuged at 1700rpm for 5 minutes. The supernatant was removed and 150µl of both 1% paraformaldehyde (PFA) and FACS buffer was added to each tube to fix the cells. The cells were then analysed immediately. Analysis was performed using a Canto II flow cytometer.

Gating and compensation was performed to select CD14 positive cells for analysis of TLR4 and TLR5 cell surface expression. Compensation in flow cytometry is the process of resolving spectral overlap between different fluorochromes. It allows analysis of complex data sets provided compensation has been performed correctly and appropriate fluorochromes have been selected. Due to its spectral range, Pacific Blue does not require compensation and is therefore a useful fluorochrome. Compensation is essential when fluorochromes have similar spectral wavelengths and spill over can occur (e.g. PE and FITC). Compensation beads were suspended in 200µl FACS buffer and run with each experiment.

Samples were also tested with isotype control antibodies to ensure that CD14, CD16, TLR4 and TLR5 antibody binding was specific (Figure 2.2). TLR4 and TLR5 expression on CD14

positive cells was calculated and expressed by median fluorescence intensity (MFI) and the MFI indicates TLR4 and TLR5 expression on peripheral blood monocytes.



Figures 2.2. FACS histograms comparing isotype control (red histogram) to the specific antibody (blue) histogram for TLR4 (A) and TLR5 (B) in $CD14^+$ cells. This indicates specific binding of the TLR4 and TLR5 antibody.

The gating strategies to identify the classical and intermediate monocyte subpopulations are shown in chapter 5.

2.7 Phos-flow analysis of transcription factor phosphorylation

Antibody	Fluorchrome	Isotype control
Human anti-CD14 (Biolegend, Cambridge, UK)	Pacific blue	Mouse IgG2a
Mouse anti-NF-κB p65 (pS529) (BD Biosciences, Oxford, UK)	Phycoerythrin (PE)	Mouse IgG2b
Mouse anti-STAT1 (pY701) (BD Biosciences, Oxford, UK)	Fluorscein isothiocyanate (FITC)	Mouse IgG2a
Mouse anti-ERK1/2 (pY204) (BD Biosciences, Oxford, UK)	Alexa Fluor 647 (AF647)	Mouse IgG1

Table 2.8. Antibodies and isotype controls used in monocyte analysis.

Solutions and reagents	Equipment
Complete medium (RPMI-1640, 50mls 10%FCS, 1% L-glutamine, penicillin/streptomycin)	12 well tissue culture plate
10 µl LPS (concentration 2µg/ml) (Sigma-Aldrich, Dorset, UK)	FACS tubes
5µg Flagellin (concentration 10µg/ml) (Invivogen, Toulouse, France)	Centrifuge
Recombinant Interferon α (concentration 10,000units/ml) (Peprotech, London, UK)	P1000, P200 and P20 pipettes
Phorbol 12-myristate 13-acetate (PMA)	Pipette tips
1% Paraformaldehyde (PFA)	Canto II (BD Biosciences, Oxford, UK)
Perm buffer III (BD Biosciences, San Jose, USA)	
PBS	
FACS buffer (500mls PBS, 10mls Foetal calf serum (FCS), 0.1g sodium azide, 0.18g EDTA)	

Table 2.9. Equipment required for phosflow analysis of inflammatory transcription factors.

2.7.1 Quantification of NF-κB, ERK1/2 and STAT1 phosphorylation in CD14 positive cells (monocytes)

To quantify transcription factor phosphorylation, complete medium was warmed in a water bath at 37°C for 15 minutes. PBMCs were removed from the freezer and once thawed, were centrifuged at 1700rpm for 5 minutes. The resulting pellet was re-suspended in 1ml of complete medium and the cells counted using a haemocytometer. 2 x 10^6 PBMCs were then re-suspended in 1ml of complete medium and put into a 12 well plate. The plate was incubated at 37°C (5% CO_2) for 2 hours.

Following incubation, the cells were aspirated from each well and put into labelled FACS tubes. Each well was washed with 1ml of complete medium to ensure complete removal of any cells that had adhered to the base of the tissue plate well and aspirated into the FACS tubes. There were 6 labelled FACS tubes for each sample of PBMCs, these were labelled as;

1. Unstimulated isotype control
2. Unstimulated
3. Flagellin stimulated
4. LPS stimulated
5. Interferon stimulated
6. PMA stimulated

These were then centrifuged at 1700rpm for 5 minutes. The medium was discarded and the cells re-suspended in 200µl of complete medium. The labelled FACS tubes were then stimulated with the corresponding agonist; 2µl flagellin (10µg/ml), 10µl LPS (2ng/ml), 20µl IFNα (10,000units/ml) and PMA (40mM). The FACS tubes were then vortexed briefly and placed in a water bath at 37°C for 15 minutes. Following the incubation, 200µl of warmed

1% PFA was added to each tube to fix the cells. These were vortexed and incubated in the water bath at 37°C for 10 minutes. At this point, Perm buffer III (BD Biosciences, San Jose, USA) was aliquoted into a 15ml Falcon tube and stored at -20°C. Following the incubation, 2mls of PBS were added to each FACS tube and centrifuged at 1700rpm for 5 minutes. The supernatant was poured off and 1ml of Perm buffer III was added to each tube. This was stored on ice in the dark for 30 minutes.

Following the incubation on ice, each FACS tube was centrifuged at 1700rpm for 5 minutes. The Perm buffer III was aspirated and the PBMCs were re-suspended in 2mls of FACS buffer and centrifuged at 1700rpm for 5 minutes. This process was then repeated and the FACS buffer was removed. The antibodies (STAT1, NF-κB, ERK1/2) were then added to each tube and vortexed. The PBMCs were incubated at room temperature for 45 minutes in the dark. Each tube was then washed with 2mls of FACS buffer and centrifuged at 1700rpm for 5 minutes. The FACS buffer was aspirated and each tube re-suspended in 300µl of FACS buffer and immediately analysed on a Canto II FACS machine.

Analysis was performed using a Canto II flow cytometer. Gating and compensation was performed to select CD14 positive cells for analysis of NF-κB, STAT1 and ERK1/2 phosphorylation. Each experiment was performed with an unstimulated isotype control, to confirm specific antibody binding in the samples. The data was analysed using WinList™ (Verity Software House) and was expressed as the percentage increase in phosphorylation above background.

2.8 Healthy PBMCs

Preparing pooled PBMCs	Other equipment
Water bath at 37°C	Centrifuge
Complete medium (RPMI-1640, 50mls 10%FCS, 1% L-glutamine, penicillin/streptomycin)	12-well tissue culture plate
15ml Falcon tube	1.8ml cryopreservation tube
Haemocytometer	
Microscope	
10% DMSO Freezing mixture	

Table 2.10. Equipment required for pooling of PBMCs.

Cryopreservation tubes containing frozen PBMCs from all healthy controls were removed from the -80°C freezer and incubated in the water bath for 15 minutes. PBMCs from each healthy control were transferred to separate labelled falcon tubes and centrifuged at 1700rpm for 5 minutes. The freezing mixture was discarded and each cell pellet was re-suspended in 1ml of complete medium. Each sample of PBMCs was counted in turn. 5×10^6 PBMCs from each healthy control were counted and pooled in 5ml of complete medium. This mixture was then aliquoted into five separate falcon tubes and centrifuged at 1700rpm for 5 minutes. The medium was discarded from each tube and each of the cell pellets were re-suspended in 10% DMSO freezing mixture and placed in a Mr Frosty® at -80°C.

2.9 Serum conditioning of healthy control PBMCs

Thawing the pooled PBMCs	Agonist stimulation
Water bath at 37°C	LPS (stock solution 2mg/ml)
Complete medium (RPMI-1640, 50mls 10%FCS, 1% L-glutamine, penicillin/streptomycin)	Flagellin (stock solution 1mg/ml)
15ml Falcon tube	12-well tissue culture plate
Haemocytometer	Patient serum
Microscope	

Table 2.11. Equipment required for serum conditioning of pooled PBMCs.

An aliquot of pooled PBMCs was removed from the -80°C freezer and placed in the water bath for 15 minutes. Once thawed, this was centrifuged at 1700rpm for 5 minutes. The DMSO freezing solution was discarded and the cell pellet was re-suspended in complete medium. The cells were then counted using a haemocytometer and 2 x 10^6 PBMCs were re-suspended in 1ml of complete medium and pipetted into each well of a labelled 12 well plate. 500µl of patient serum was added to each well and incubated for 24 hours (figure 2.7). The serum-conditioned PBMCs were then either left unstimulated, stimulated with 10µl (working concentration 2ng/ml) of LPS or stimulated with 2µl (working concentration 2mg/ml) of flagellin. The plate was then incubated at 37°C for 24 hours at 5% CO_2.

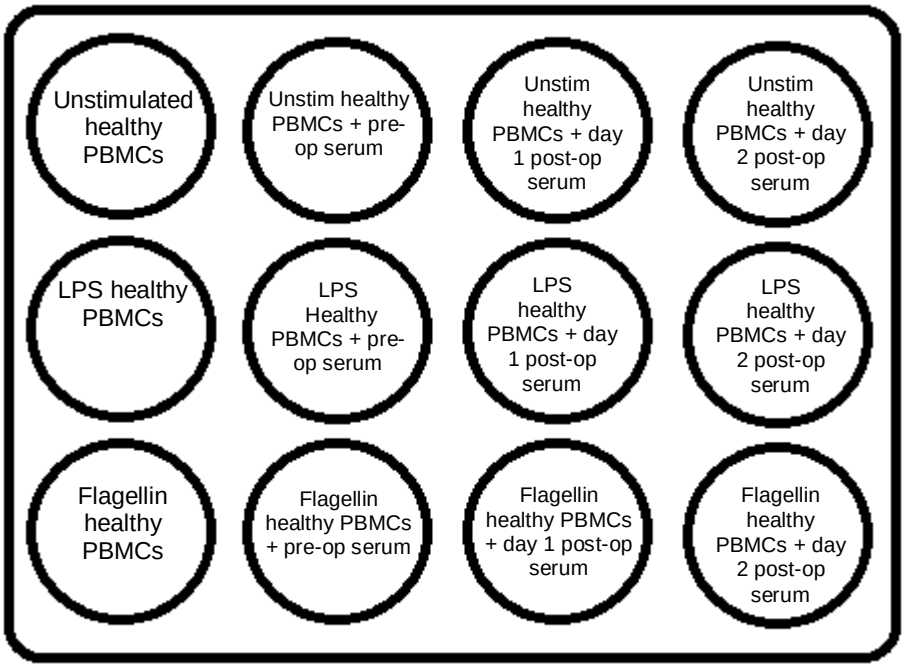


Figure 2.3. An example of a 12 well plate containing serum-conditioned healthy PBMCs alone, and stimulated pooled PBMCs and patient serum for a single patient. Unstim – unstimulated. LPS – lipopolysaccharide.

Following incubation, the PBMCs were aspirated from each well. 1ml of complete medium was added to each well to ensure removal of any cells adhered to the base of the tissue culture plate. The cells were centrifuged at 1700rpm for 5 minutes and the supernatants were removed. Supernatants were stored in eppendorfs at -20°C until required for ELISA. The PBMCs were then used for flow cytometry as previously described. A flow diagram simplifying the methods used in this thesis is shown in Figure 2.3.

2.10 Statistical analysis

Given that the role of PBMC cytokine production, TLR4, TLR5 expression and transcription factor phosphorylation in post-operative SIRS has not been previously evaluated in the literature, a power calculation was not performed to determine a sample size for the cohort. This is due to the fact that I did not know whether there would be any difference in the experimental variables evaluated in this thesis.

All data presented in the results section was tested for normality using the D'Agostino Pearson test. Continuous data was analysed using appropriate statistical tests (unpaired t test, Mann Whitney test) based on normality of distribution. Contingency testing (χ^2 test) was used when appropriate.

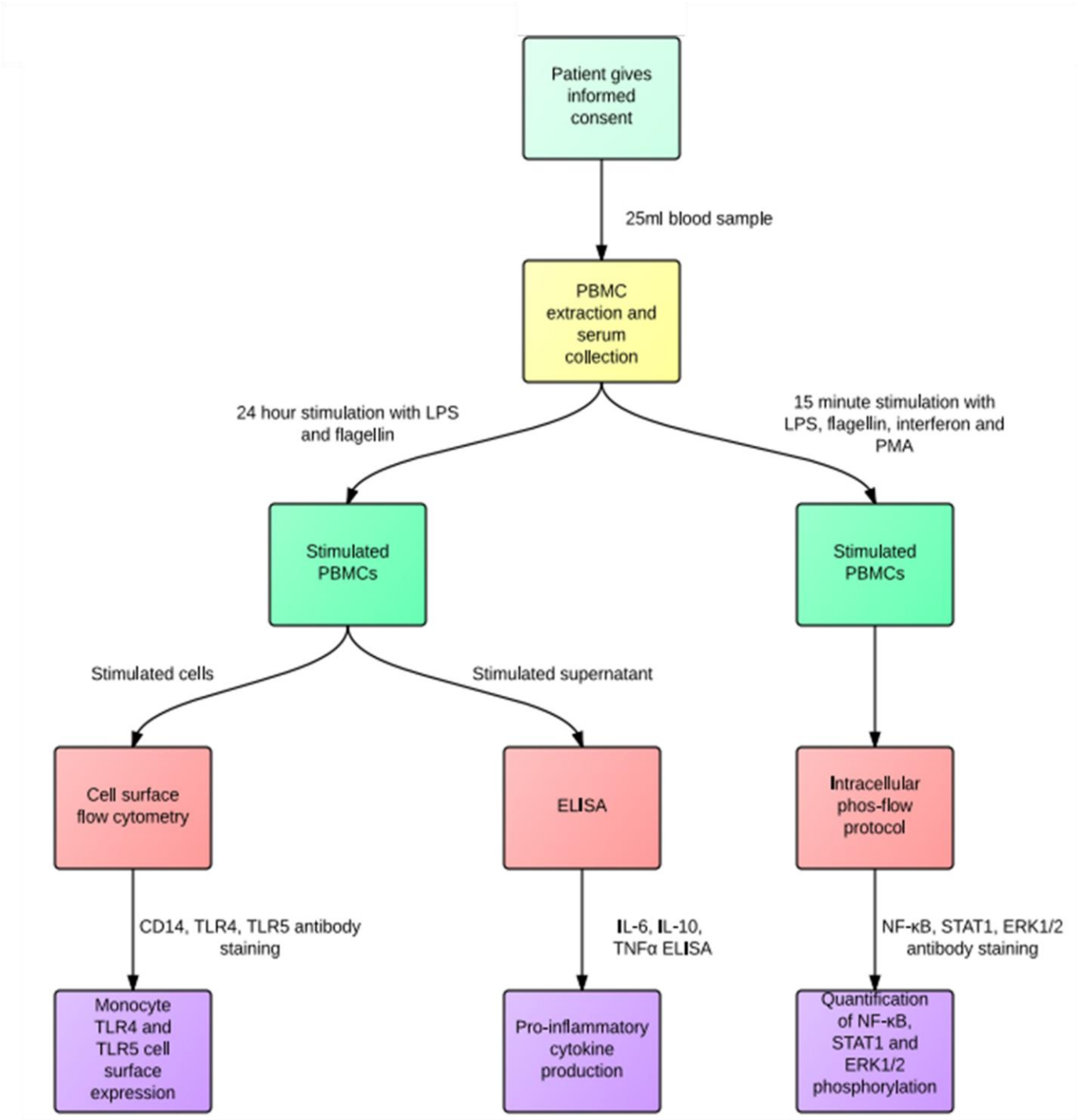


Figure 2.4. A flow diagram to demonstrate the protocols used in this thesis.

3. Evaluation of routine peri-operative parameters as predictors of post-operative SIRS

3.1 Introduction

We tested the hypothesis that markers of innate immune dysfunction can predict systemic inflammatory response syndrome (SIRS) in patients who have undergone complex hepatic, pancreatic and biliary (HPB) surgery. Patients were recruited from the HPB department at The Royal London Hospital, part of Barts Health NHS Trust, which is the largest NHS trust in the United Kingdom. The HPB department includes four consultant surgeons, junior medical staff, nurse specialists and is supported by interventional radiology and intensive care. Following major hepatic or pancreatic resection, all patients are nursed on the high dependency unit (HDU). This allows more intensive nursing and invasive monitoring in the early post-operative period and is part of the standard clinical care. In this chapter I present the clinical characteristics of the study cohort and detail the operations that the patients underwent. I show that routinely collected clinical or pathological data could not predict the development of SIRS in our cohort.

3.1.1 Patient cohort

39 patients agreed to participate and were consented on the morning of surgery. Each patient was given a patient information sheet prior to consenting. The demographics, co-morbidities and operation performed in the study cohort are shown in Table 3.1.

Patient number	Age	Sex	Indication	Co-morbidities	Medication	Operation
1	60	M	Duodenal obstruction	Previous laparotomy for obstruction (2006)	-	PPPD
2	74	F	Mucinous mass in head of pancreas	Stroke, HTN, hypercholesterolaemia, AAA under surveillance	Gabapentin, lansoprazole, dipyramidole, atorvastatin, bendroflumethiazide	PPPD
3	53	F	Mass at head of pancreas	-	-	PPPD
4	66	F	Mass at head of pancreas	Anal cancer 2001 (in remission)	-	PPPD
5	74	F	Painless jaundice, mass at head of pancreas	DM	-	PPPD
6	85	M	Weight loss, early satiety	IHD, HTN, BPH	Aspirin, atenolol, tamsulosin, simvastatin, ramipril, lansoprazole, finesteride, GTN	PPPD
7	70	F	Painless jaundice, mass at head of pancreas	-	-	PPPD
8	66	F	Late onset DM and jaundice	Varicose veins, DM	Gliclazide	PPPD
9	69	M	Abdominal pain and weight loss	Hypercholesterolaemia, Hypothyroidism	Simvastatin, Levothyroxine	PPPD
10	39	M	IPMN in head of pancreas	Recurrent pancreatitis, GORD	Omeprazole	PPPD
11	48	M	Head of pancreas PDAC	-	-	PPPD
12	44	F	Mass at head of pancreas	-	-	PPPD
13	66	M	Mass at head of pancreas	-	-	PPPD
14	52	F	Incidental mass at tail of the pancreas	Bilateral mastectomies + BSO	Letrozole	DP
15	80	M	Obstructive jaundice	Thyroidectomy	Levothyroxine	PPPD
16	77	F	Colorectal liver metastasis	HTN, right hemicolectomy (2009)	Perindopril	Right hemi-hepatectomy
17	70	F	Colorectal liver metastases	Anterior resection (2008) complicated by ileus	-	Right hemi-hepatectomy
18	22	F	Type I choledochal cyst	-	-	CBD excision and Roux-en-Y reconstruction
19	65	M	Colorectal liver metastases	Anterior resection (2010)	-	Left hemi-hepatectomy
20	60	M	Colorectal liver metastasis	Anterior resection (2005),	Simvastatin	Extended right hemi-

				hypercholesterolaemia		hepatectomy
21	55	M	Duodenal tumour	OSA, HTN, AF	Ramipril, Warfarin	PPPD
22	60	M	Mass at head of pancreas	-	-	PPPD
23	63	M	Renal cell cancer recurrence in the head of the pancreas	Renal cell cancer. Right nephrectomy in 1996.	-	PPPD
24	69	M	Body of pancreas tumour	AF, HTN, hypercholesterolaemia	Warfarin, Perindopril, Simvastatin	DP
25	38	F	Mucinous cystic neoplasm at tail of pancreas	BMI 51	-	DP
26	71	F	Mass at head of pancreas	Hypercholesterolaemia	-	DP
27	68	F	Neuroendocrine duodenal tumour	IHD, HTN, Depression	Aspirin, rampril, citalopram	PPPD
28	72	M	Extra-hepatic cholangio-carcinoma	-	-	PPPD
29	77	M	Mass at head of pancreas	CRF, hypothyroidism	Levothyroxine, Amlodipine	PPPD
30	63	F	Metastatic carcinoid tumour in liver	Depression, hypercholesterolaemia	Paroxetine, Atorvastatin	Right hemi-hepatectomy
31	74	F	Mass at head of pancreas	-	-	PPPD
32	63	M	Mass at head of pancreas	HTN, stroke	Aspirin, ramipril	PPPD
33	53	M	Cystic mass in body of pancreas	TURBT	-	DP
34	82	F	Colorectal liver metastasis	Anterior resection (2012), HTN, hypercholesterolaemia	Simvastatin, Amlodipine	Right hemi-hepatectomy
35	82	F	Mass at head of pancreas	Previous puestow operation, HTN, Bipolar disorder	Amlodipine, Ramipril, Lithium	PPPD
36	67	M	Recurrent colorectal liver metastases	HTN, Anterior resection (2009) and wedge resection of liver tumour	Ramipril	Right hemi-hepatectomy
37	66	M	Mass at head of pancreas	Previous right shoulder surgery, left inguinal hernia repair. HTN	Amlodipine	PPPD
38	77	M	Colorectal liver metastasis	Extended right hemicolectomy (2011), HTN	Amlodipine	Left hemi-hepatectomy
39	66	F	Intra-hepatic cholangio-carcinoma in left liver	Hiatus hernia	Omeprazole	Left hemi-hepatectomy

M – male, F – female, - - no complications DM- diabetes mellitus, IPMN – intraductal papillary mucinous neoplasm, PDAC – pancreatic ductal adenocarcinoma, Mets – metastases, HTN – hypertension, AAA – abdominal aortic aneurysm, IHD – ischaemic heart disease, BPH – benign prostatic hypertrophy, ERCP –

endoscopic retrograde cholangiopancreatography, GORD – gastro-oesophageal reflux disease, OSA – obstructive sleep apnoea, BSO- bilateral salphingo-oopherectomy, AF – atrial fibrillation, BMI – body mass index, CRF – chronic renal failure, TURBT – transurethral resection of bladder tumour, PPPD – pylorus preserving pancreaticoduodenectomy, DP – distal pancreatectomy.

Table 3.1. The demographics, indication, past medical history, drug history and operative procedure of all study patients.

The mean age of the patients was 63.2 years (range 22-85). There were 20 men (52%) and 19 women (48.8%) included in the study. There was no significant difference in mean age between men and women in the study group. (63.9 years versus 62.4 years; p=ns). 37 patients were Caucasian (95.2%), one patient was of Bangladeshi (2.4%) and one patient of Indian (2.4%) ethnicity.

12 patients developed SIRS following surgery (see 3.1.3) and there was no significant difference in the incidence of post-operative SIRS between the sexes. In addition, there was no significant difference in age, co-morbidities or medication history between patients who developed SIRS and those who had an uneventful recovery when the patients were analysed by procedure. In addition, there was no significant difference in the incidence of SIRS between cases performed for malignant versus benign disease. In patients who underwent PPPD, there was no difference in the incidence of biliary stenting between the SIRS and non-SIRS groups.

3.1.2 Pre-medication and anaesthetic approach

Prior to all pancreatic cases, each patient was given 100 micrograms of Octreotide subcutaneously. Octreotide is a somatostatin analogue that is commonly used to reduce pancreatic secretions and it was continued for 3 days post-operatively. All patients taking anticoagulants stopped taking them five days before surgery. Each HPB consultant works

with a regular consultant anaesthetist and there was no difference in the anaesthetic regime (propofol for induction, sevoflurane for maintenance) used for each patient. There was no significant difference in the use of epidural analgesia between patients who have an uneventful recovery and those who developed SIRS. All patients undergoing major hepatic or pancreatic resection were given broad spectrum intravenous antibiotics (intravenous co-amoxiclav) at induction and two post-operative doses in line with local guidelines. All patients had a central venous catheter (CVC) and arterial line inserted for invasive monitoring during the anaesthetic.

3.1.2.1 Intra-operative details of patients undergoing pylorus preserving pancreaticoduodenectomy

24 patients were scheduled for pancreaticoduodenectomy. Of these, 22 patients underwent pylorus-preserving pancreaticoduodenectomy (PPPD). One patient had unresectable disease at laparotomy and underwent palliative bypass with cholecho-jejunostomy and gastro-jejunostomy. All procedures were performed by one of three consultant HPB surgeons. There was no significant difference in the incidence of post-operative SIRS between the surgeons. All three surgeons used a similar operative approach in all cases. No patients required portal vein resection and vascular reconstruction. The median operative time was 320 minutes (range 230-585 minutes). There was no significant difference in operative time between patients who have an uneventful recovery and those who developed SIRS. The patient whose PPPD took almost 10 hours had dense adhesions in the upper abdomen following previous surgery. Median intra-operative blood loss was 810ml (range 340-5050ml). There was no significant difference in intra-operative blood loss between the groups. In addition, there was no significant difference in the use of intra-operative red blood cell transfusion in patients undergoing PPPD. The histological and staging details for all patients are in Appendix 1. There was no significant difference in the incidence of malignant disease or tumour stage between patients who develop post-operative SIRS and those who had an uneventful recovery. Instrumentation of the bile duct (e.g. stent

insertion) did not increase the risk of developing post-operative SIRS and only this would potentially introduce infection into the bile duct pre-operatively.

3.1.2.2 Intra-operative details of patients undergoing distal pancreatectomy

4 patients underwent open distal pancreatectomy (DP) and splenectomy of whom two patients had malignant disease. Concurrent splenectomy was performed to ensure adequate oncological clearance. In the 2 benign cases, the spleen was removed as the cystadenoma was very large and the case of IPMN had malignant features on pre-operative imaging. Both patients who developed SIRS following DP were male. In all cases, the remnant pancreas was closed with a stapling device and over sewn with a 4.0 prolene suture to reduce the rate of pancreatic leak. The splenic hilum was transected using a vascular stapler following arterial clamping to reduce the volume of blood in the spleen prior to hilar transection. The median operative time for open distal pancreatectomy was 245 minutes (range 180-305 minutes) and median intra-operative blood loss was 350ml (150-950ml). There was no significant difference in intra-operative blood loss or use of blood products between the two groups. The histological and staging details are in Appendix 1. There was no significant difference in the incidence of malignant disease between patients who develop post-operative SIRS and those who had an uneventful recovery.

3.1.2.3 Intra-operative details of patients undergoing major liver resection

Nine patients underwent major liver resection, of whom eight had metastatic colorectal cancer confined to the liver. The other patient had a metastatic neuroendocrine tumour. All patients with liver metastases secondary to colorectal cancer had neoadjuvant

chemotherapy prior to liver resection as decided at the HPB MDT. Six patients underwent right hemi-hepatectomy of whom one underwent trisegmentectomy (removal of segments 4-8) following hepatic artery embolisation. The remaining three patients had left hemi-hepatectomies. There was no significant difference in the incidence of SIRS between the sexes. All patients had open resections. None of the patients included in the study required inferior vena cava (IVC) clamping or major venous resection. Median operative time was 210 minutes (140-290 minutes) and median blood loss was 790ml (300-3200ml). There was no significant difference in intra-operative blood loss or use of blood products between patients who have an uneventful recovery and those who develop SIRS. The histological and staging details for are in Appendix 1.

Table 3.2 below summarises the fact that there was no difference between patients that develop SIRS and those that do not in terms of intra-operative variables. The operative factors were combined for all operations.

	No SIRS		**SIRS**		**P value**
Sex	Male 14/20	Female 13/19	Male 6/20	Female 6/19	p=ns
Ethnicity	Caucasian 25/37	Other 1/2	Caucasian 12/37	Other ½	p=ns
Operative Factors					
Operative time (min)	270 (135-460)		323 (170-585)		p=ns
Blood loss (ml)	773 (190-2010)		1088 (150-3200)		p=ns
Intra-operative transfusion (n(%))	7 (26)		6 (50)		p=ns

Table 3.2. Demographic and intra-operative details

3.2 Diagnosing patients with SIRS

Patients were diagnosed with SIRS according to the internationally agreed criteria outlined in chapter 1.2.1. In total, 12 patients (30.7%) were diagnosed with SIRS in the post-operative period. The median day on which SIRS was diagnosed was day 6 (range 2-12). There was no significant difference in age or sex between patients who developed SIRS and those who had an uneventful recovery. The cause of SIRS is shown in Table 20. Of the 12 patients, 2 died within 30 days of surgery. The cause of death is also shown in Table 3.3.

There was no difference in incidence of post-operative SIRS and sepsis between consultant surgeons. The majority of patients (73%) with post-operative complications had undergone major pancreatic surgery. There was no significant difference between the incidence of SIRS in patients undergoing pancreatic surgery and hepatic surgery (34.3% and 22.2%; $p<0.2$). The median day on which patients developed SIRS was 6 days (range 2-12). The median length of time to discharge was 22 days (range 12-52) in patients who developed post-operative SIRS and 13 days (range 6-24) in those with an uneventful recovery ($p<0.0002$). The cause and treatment of SIRS in patients following surgery is shown below.

Patient number	Operation	Day SIRS criteria met	Cause	Management	Discharge
2	PPPD	6	LRTI	IV antibiotics and transferred to HDU for CPAP	27
4	PPPD	8	Infected intra-abdominal collection	IV antibiotics. Did not require further drainage	16
5	PPPD	6	LRTI	Oral antibiotics	14
7	PPPD	5	Infected post-operative collections	IV antibiotics and radiological drainage. Returned to HDU for supportive care	52
20	Excision of choledochal cyst	2	No identified source	Supportive measures. No evidence of sepsis	12
22	Right hemi-hepatectomy	6	Liver abscess	IV antibiotics. Radiological drain inserted.	18
26	DP	6	LRTI	IV antibiotics. Chest physiotherapy	15

31	PPPD	4	Infected post-operative collection	IV antibiotics. Radiological drain inserted.	20
33	PPPD	12	Bibasal LRTI	IV antibiotics. Chest physiotherapy	22
36	Right hemi-hepatectomy	10	LRTI	IV antibiotics. Chest physiotherapy	19
Deaths					
Patient number	**Operation**	**Day SIRS criteria met**	**Cause**	**Management**	**Death**
23	PPPD	2	ARDS secondary to pancreatic leak	IV antibiotics and ventilator and dialysis support. Re-look laparotomy on POD 3.	4
24	PPPD	2	Grade C pancreatic leak leading to SMA thrombosis and entire small bowel ischaemia.	Re-look laparotomy. Degree of damage to small bowel unsalvageable.	3

LRTI – lower respiratory tract infection, IV – intravenous, CPAP – continuous positive airway pressure, ARDS – adult respiratory distress syndrome, SMA – superior mesenteric artery, POD – post-operative day.

Table 3.3. Clinical details of 12 patients who developed SIRS following surgery.

Two patients died following surgery. Both were male and underwent PPPD. The length of operation in these patients was not significantly longer than in the other patients in the study. There was also no significant difference in blood loss between the two patients who died and all other patients in the study. Both patients were admitted to HDU following surgery before being transferred to the intensive therapy unit (ITU) after deteriorating. Both patients returned to theatre for re-look laparotomy before they died. The cause of death and immediate treatment is shown in Table 3.3.

3.3 Peri-operative haematology and biochemistry

There was no significant difference in pre-operative haemoglobin, white cell count (WCC) or platelets between patients who had an uneventful recovery and those who developed post-operative SIRS. In addition to pre-operative haematology, routine biochemistry was

performed on all patients. There was no difference in pre-operative Creatinine, Urea or Bilirubin levels between patients with an uneventful recovery and those that developed post-operative SIRS (Table 3.4).

Post-operatively, there was no significant difference in haematological (haemoglobin, WCC, platelets) or biochemistry (urea, creatinine, bilirubin, CRP) between patients who had an uneventful recovery and those who develop SIRS on day one or day two (Table 3.4). Haemoglobin was lower and WCC raised postoperatively compared to pre-operatively and C-reactive protein (CRP) was significantly greater on day two compared to day one ($p<0.02$). However, these parameters were not significantly different in patients who developed SIRS compared to those who did not. There was no significant change in urea, creatinine and bilirubin on post-operative day one and two compared to pre-operative values.

Clinical parameter	SIRS (mean+range)	No complications (-) (mean+range)	p value
Pre-operative values			
Hb	13.1 (12-14.5)	12.8 (11-14.5)	p=ns
WCC	8.1 (5.2-10.4)	7.9 (5.2-10)	p=ns
Plts	274 (138-411)	265 (129-402)	p=ns
Ur	5.1 (3.5-8.4)	4.8 (3.8-8.6)	p=ns
Cr	79 (59-102)	81 (48-147)	p=ns
Bili	18 (0-48)	12 (0-31)	p=ns
Post-operative day one			
Hb	10.1 (8.6-12.5)	10.2 (9.0-12.5)	p=ns
WCC	13.1 (9.2-15.1)	14.1 (8.0-17.9)	p=ns
Plts	221 (104-442)	210 (98-429)	p=ns
Ur	6.6 (4.4-10.2)	6.9 (3.9-10.5)	
Cr	75 (49-100)	81 (41-143)	p=ns
Bili	18 (3-48)	15 (8-30)	p=ns
CRP	49 (21-76)	52 (5-108)	p=ns
Post-operative day two			
Hb	9.6 (7.9-13.0)	9.4 (7.8-12.8)	p=ns
WCC	14.1 (7.5-20.3)	14.5 (7.8-24.1)	p=ns
Plts	202 (99-400)	198 (83-389)	p=ns
Ur	6.3 (3.4-8.8)	6.2 (3.3-8.5)	p=ns
Cr	81 (48-167)	78 (41-150)	p=ns
Bili	14 (4-31)	15 (4-46)	p=ns
CRP	111 (23-203)	118 (48-257)	p=ns

Table 3.4. Pre-operative and day one and day two haematology and biochemistry.

3.4 Discussion

In this study cohort, no demographic, medical, anaesthetic or surgical data could predict patients at high risk of developing SIRS following major HPB surgery in the early post-operative period. In addition to this, routine haematological and biochemistry taken pre-operative and post-operative days one and two could not distinguish between patients who had an uneventful recovery and those who went on to develop SIRS.

3.4.1 Demographic and medical factors do not predict post-operative SIRS

As stated in 3.1.1, there was no significant difference in age between patients who have an uneventful recovery and those who develop SIRS. A previous study found that elderly patients (over 75 years old) undergoing surgical stress had significantly greater monocyte activation and pro-inflammatory cytokine production compared to younger patients (21). The duration of SIRS was also significantly greater in the elderly group. However, this group did not evaluate the potential effect of co-morbidities and did not look at any differences in haematology and biochemistry between the groups. A retrospective study identified that elderly patients requiring emergency abdominal surgery had a higher mortality rate than younger patients (157). The results in this study do not acknowledge that during the study period (1983 to 1997), that post-operative intubation and ITU care was less common than it is now in elderly patients. Another major difference is that patients requiring emergency abdominal surgery often meet SIRS criteria on admission. The high risks associated with major HPB surgery and the numerous co-morbidities that many elderly patients have could lead to the decision not to offer surgery to high risk elderly patients following MDT discussion thereby contributing to the relatively young mean age (63.2 years) of the cohort in this study. In addition to patient selection, recent guidelines from the National Confidential Enquiry into Patient Outcome and Death (NCEPOD: An age old problem) state that daily input from care of the elderly physicians and ITU support following major surgery

can improve outcomes and this is implemented in all elderly patients undergoing HPB surgery at Barts Health NHS Trust.

There were also no differences in gender between patients who had an uneventful recovery and those who developed SIRS in this study. There was no difference in the age of the men and women who developed SIRS following surgery (158). In contrast, another study demonstrated that men produced more inflammatory cytokines in response to LPS than women (159). A large clinical cohort study determined that fewer female surgical patients were referred and admitted to ITU and that fewer females developed severe sepsis (160). This suggests that women may be less susceptible to post-operative severe sepsis in a large series of patients undergoing a variety of abdominal operations. However, we did not see any differences between the rates of SIRS in males versus females in our cohort of patients undergoing complex HPB surgery. Although there are numerous reports supporting differences in immune function between the sexes, the lack of significant difference seen in this study can most readily be explained by the fact that 12 patients developed SIRS and this may be too few to illicit a difference in the early post-operative period. A strength of our study was that there was no difference in age, co-morbidities, drug history, anaesthetic protocol, surgical procedure and post-operative haematology and biochemistry between male and female patients indicating that there are no other clinical factors that may be skewing these results.

To limit the effect of co-morbidities on the experimental design, patients with inflammatory co-morbidities (e.g. Crohns disease, Rheumatoid arthritis), pre-operative sepsis and patients taking regular anti-inflammatory medications were excluded from the study. This was done as these factors have been shown to influence TLR expression. (161-163).

3.4.2 Anaesthetic or surgical factors did not predict post-operative SIRS

In this cohort, the same anaesthetic regime was used for each patient. All patients in this study had propofol for induction and sevoflurane for maintenance during surgery. Evaluation of the role of anaesthetic agents in post-operative SIRS has been investigated. A randomised trial in Greece has demonstrated no significant difference in the incidence of SIRS following major cardiac surgery between patients receiving total IV anaesthesia using propofol and patients receiving propofol for induction and sevoflurane for maintenance (165). However, it is important to note that this was an interim analysis with only 40% of the total cohort analysed. A small experimental study in healthy volunteers demonstrated that propofol inhibited IL-6 and IL-10 production following LPS stimulation of PBMCs (166). However, the group used submaximal dose of LPS (3pg/ml) and the changes in both IL-6 and IL-10 production from baseline were very small. In addition to this, one of the higher doses of propofol did not inhibit IL-6 and IL-10 production and the study did not address the mechanism for any potential inhibitory effect.

There was no difference in intra-operative blood transfusion or blood loss between patients who had an uneventful recovery and those who developed SIRS in this study. A retrospective analysis of trauma patients identified that patients who received a blood transfusion in the first 24 hours following trauma had a higher incidence of SIRS (167). However, these patients were significantly older and had lower Glasgow Coma Scores (GCS) indicating more significant trauma. Also, the nature of trauma is different to elective surgery, so the findings cannot be extrapolated directly. Another study found that patients who developed SIRS following orthotopic liver transplantation (OLT) had required significantly more intra-operative blood transfusion and had significantly greater blood loss than those who did not develop SIRS (15). OLT is known to be a procedure with significant blood loss and many of these patients have clotting abnormalities, which may contribute to the development of SIRS. An abstract presented at the American Heart Association meeting

(Stocker et al. Circulation 2007) in 2007 summarised a randomised trial in children undergoing elective cardiac surgery found that there was no difference in blood loss between patients who developed SIRS and those who did not. A study in patients undergoing liver surgery found that larger liver resections had greater blood loss and post-operative complications (168). However, both of these findings are to be expected given the surgical trauma of hepatic surgery and the known complications of hepatic insufficiency following extended hepatectomies. A study comparing standard pancreaticoduodenectomy (PD) to radical PD found that although post-operative complications were more common following radical PD, there was no difference in blood loss between the procedures (169). Therefore, it has not yet been proven definitively that intra-operative blood loss is an independent risk factor for the development of SIRS. One factor that was not recorded intra-operatively was the length of time that the common bile duct (CBD) was left open prior to reconstruction during PPPD. It is possible that increased bile spillage may contribute to post-operative SIRS.

3.4.3 Peri-operative haematological and biochemistry investigations did not predict post-operative SIRS

In this study, no routine haematological or biochemical markers measured pre- or post-operatively were significantly different between patients who develop SIRS and those who have an uneventful recovery. A study looking a post-operative changes in routine investigations following colorectal surgery found that WCC did not differentiate between patients who developed SIRS and those who did not (170). However, they did identify that patients who developed post-operative complications had greater CRP following surgery. These CRP findings are in contrast to those presented in this study. A study evaluating the role of post-operative CRP in predicting anastomotic leak following colorectal resection found that a CRP value of greater than 125mg/L on day 4 had a sensitivity of 81.8% and negative predictive value (NPV) of 95.8% for the detection of anastomotic leak (171). However, in contrast, another study found that day 4 CRP following major colorectal surgery

was less effective at identifying anastomotic leak (sensitivity 61.2%) (172). Interestingly, another study in 56 patients undergoing major abdominal surgery found that CRP was unable to distinguish patients who developed SIRS from those who did not (173). The cohort in the study performed by Mokart et al was similar to that in this study. In patients undergoing oesophagectomy, CRP did not identify patients with post-operative complications until day 3 (64) which was after other variables studied including IL-6. In contrast to the findings in the colorectal studies, patients who underwent liver resection and developed post-operative liver failure had significantly lower CRP values (174). In addition to Upper GI surgery, early post-operative CRP values did not predict complications in patients who had undergone cardiac bypass surgery (175). All of the above studies showed that WCC did not identify patients who developed post-operative SIRS. Taken together, this indicates that colonic mobilisation may lead to increased bacterial translocation which increases CRP more than upper GI or HPB dissection. This suggests that CRP may be a more useful predictive tool in colorectal surgery. The data presented in this chapter supports reports in the literature that WCC and CRP are not useful predictors following major HPB surgery.

In summary, the cohort of patients studied in this thesis demonstrated no patient factors, co-morbidities, anaesthetic agents, intra-operative features or early routine post-operative investigations that could identify patients at high risk of developing post-operative SIRS after complex HPB surgery. Although the cohort in this thesis is not large (39 patients), the patients are relatively homogeneous in terms of the surgical insult they received. The variability is also minimised by the fact that all operations were performed by four consultant surgeons within the same department. As described above, many studies attempting to identify pre-operative markers of post-operative complications have looked at a wider variety of surgical subspecialties. Whilst this has the advantage of increasing patient numbers, the wider variety of surgical procedures performed can lead to selection bias. As described in 3.1.5.1, strict inclusion criteria were employed as pre-existing inflammatory conditions (161), (162) have been shown to influence TLR expression. This further explains the size of the cohort in this thesis.

4. Cytokine quantification before and immediately after major HPB surgery

4.1 Introduction

ELISA is a commonly used technique to quantify cytokine production. It has numerous advantages. ELISA is known to be highly sensitive and specific and validated commercial ELISA kits are available that are relatively easy to use and inexpensive. In addition, 96 well plates allow high throughput analysis with multiple samples analysed within the same experiment. It has been validated for the use specified in this thesis. A major benefit of commercially available ELISA is that it is a convenient and well-validated tool with a wide detection range and can therefore it can be used on samples immediately and has the ability to detect very low cytokine concentrations due to specific antibody binding. It is a relatively inexpensive technique compared to other techniques, allowing the analysis of a large number of samples.

Quantification of IL-6, TNF-α and IL-10 were studied as these cytokines are known be involved in the pathogenesis of SIRS. Although the pathophysiology of SIRS is complex and is certain to involve more than just IL-6, TNF-α and IL-10, it was felt that analysis of these three cytokines would yield interesting results. This is due to the differences that have been demonstrated in previous work looking at cytokines as biomarkers for post-operative sepsis (60).

The pathophysiological process that leads to SIRS is unclear. The importance of dysregulated cytokine production in the development of SIRS was highlighted in the introduction. In addition to this, previous evidence has indicated that the production of cytokines can be dysregulated in the early post-operative period (8). I hypothesise that IL-6, TNF-α and IL-10

production by monocytes in response to key bacterial fragments, LPS and flagellin is dysregulated in patients who develop post-operative SIRS. In this chapter, I show that on post-operative day two, there was a significant increase in LPS and flagellin stimulated IL-6 (but not TNF-a or IL-10) production in patients who go on to develop SIRS compared to patients who have an uneventful recovery.

4.2. Comparison of pre- and early post-operative IL-6 production in patients who develop SIRS and those who have an uneventful recovery

IL-6 production in unstimulated pre-operative PBMCs from patients who develop SIRS, those who have an uneventful recovery (Figure 9) and healthy controls did not differ significantly. Stimulation with LPS (mean IL-6 production 409pg/ml; $p<0.004$) or flagellin (mean IL-6 production 90pg/ml; $p<0.02$) led to significantly greater IL-6 production compared to unstimulated PBMCs (mean IL-6 production 12pg/ml) for both groups of patients and healthy controls, but there were no significant differences between the groups. The same was true in PBMCs taken on post-operative day 1 (Figure 4.1).

However, on post-operative day two, there was a significant increase in IL-6 production following LPS (mean IL-6 production 1111pg/ml; $p<0.0001$) and flagellin (mean IL-6 production 892pg/ml; $p<0.0001$) stimulation in patients who went on to develop SIRS (Figure 4.1) compared to patients who had an uneventful recovery. There was no significant difference in IL-6 production by unstimulated PBMCs between the two groups of patients. The amount of IL-6 produced in response to both LPS and flagellin was significantly greater on post-operative day two compared to pre-operatively ($p<0.0001$) and post-operative one ($p<0.0002$).

Patients who developed SIRS following major HPB surgery demonstrated increased IL-6 production in response to LPS and flagellin on post-operative day two. This is four days before the median length of time to the diagnosis of SIRS using routine clinical and biochemical parameters.

4.2.1 Comparison of pre- and early post-operative TNF-α and IL-10 production in patients that develop SIRS and those who have an uneventful recovery

Interestingly, the production of TNF-α or IL-10 by the same PBMCs did not distinguish between patients who had an uneventful recovery and those who developed SIRS. There was no significant difference in unstimulated or agonist stimulated TNF-α production between patients who had an uneventful recovery and those who developed SIRS pre-operatively (Figure 4.2). Following LPS ($p<0.01$) and flagellin ($p<0.05$) stimulation, TNF-α production was significantly greater compared to unstimulated production. On post-operative days one and two, there was no significant difference in PBMC TNF-α production between patients who had an uneventful recovery and those who developed SIRS (Figure 4.2). In patients that had an uneventful recovery, there is a trend towards a daily reduction in TNF-α production following surgery.

There was a significant difference ($p<0.05$) in IL-10 production in response to LPS between patients who had an uneventful recovery (mean IL-10 production 203pg/ml) and those who developed SIRS (mean IL-10 production 340pg/ml) on post-operative day two (Figure 4.3). Pre-operatively, in both groups, LPS ($p<0.004$) and flagellin ($p<0.01$) stimulation produced significantly more IL-10 than unstimulated samples. On post-operative day one, there was no significant difference in unstimulated, LPS- or flagellin stimulated IL-10 production between patients who had an uneventful recovery and those who developed SIRS (Figure 4.3). Following surgery, stimulated IL-10 production decreased and there was significantly

less IL-10 produced in response to LPS (No SIRS pre-op 434pg/ml vs No SIRS Day Two 203pg/ml; SIRS pre-op 497pg/ml vs SIRS Day Two 340pg/ml) ($p<0.04$) and flagellin (No SIRS pre-op 402pg/ml vs No SIRS Day Two 187pg/ml; SIRS pre-op 531pg/ml vs SIRS Day Two 140pg/ml) ($p<0.01$) on day two compared to pre-operatively. There was no significant difference in peri-operative IL-10 production between those patients who developed SIRS and those who had an uneventful recovery.

In summary, early post-operative TNF-α did not distinguish between patients who had an uneventful recovery and those who develop SIRS. There was a difference in IL-10 production between the groups in response to LPS on day two following surgery.

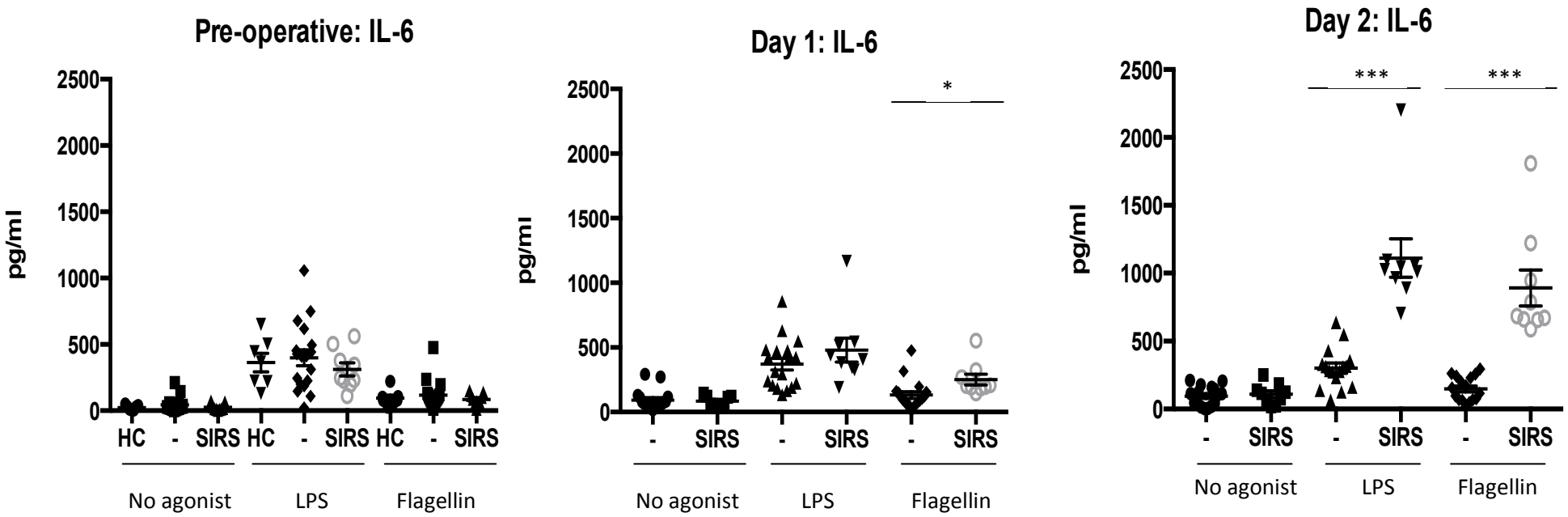


Figure 4.1. Unstimulated, LPS- and flagellin stimulated IL-6 production pre-operatively and on post-operative days one and two following surgery. IL-6 production was compared between patients who did not develop complications (-) and patients who develop SIRS (SIRS).

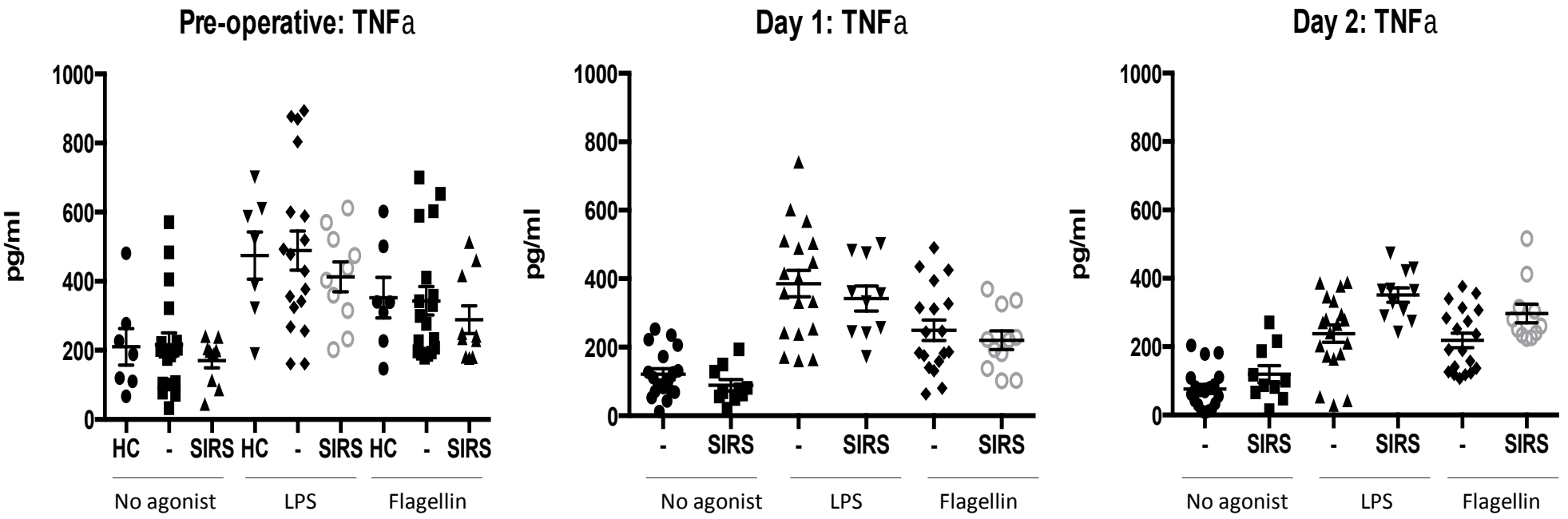


Figure 4.2. Unstimulated, LPS- and flagellin stimulated TNF-α production pre-operatively and on post-operative days one and two following surgery. TNF-α production was compared between patients who did not develop complications (-) and patients who develop SIRS (SIRS).

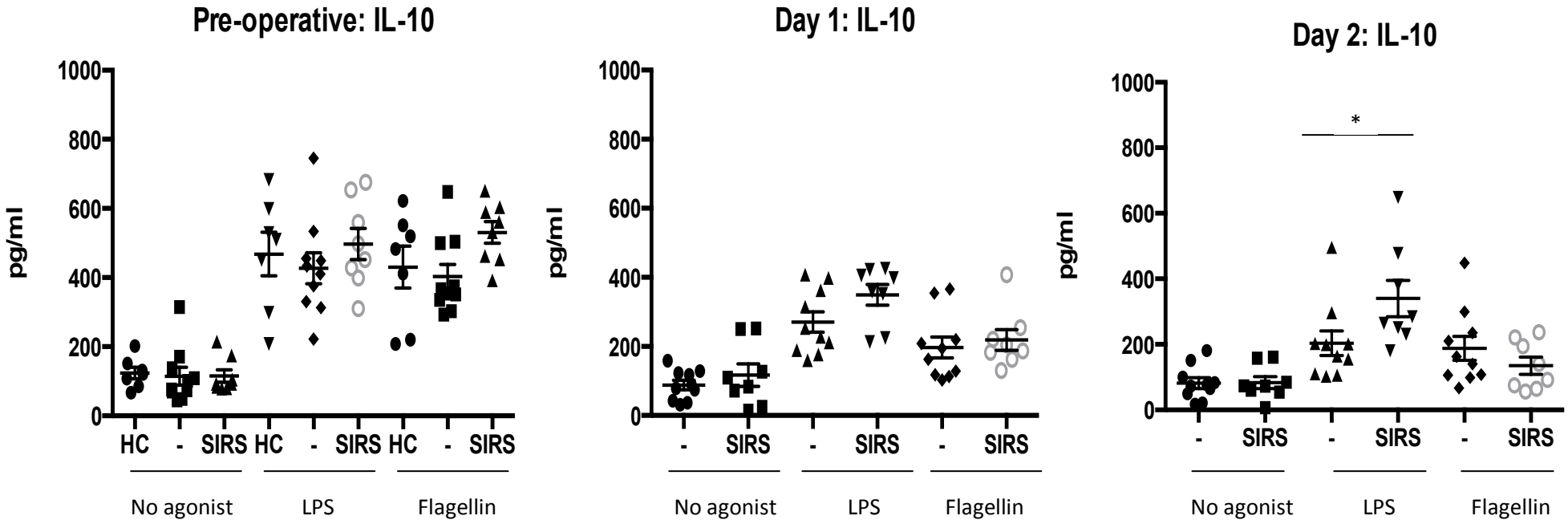


Figure 4.3. Unstimulated, LPS- and flagellin stimulated IL-10 production pre-operatively and on post-operative days one and two following surgery. IL-10 production was compared between patients who did not develop complications (-) and patients who develop SIRS (SIRS).

4.3 IL-6, TNF-α and IL-10 production in patient serum

To quantify IL-6, TNF-α and IL-10 levels in serum, serum was analysed using a commercial available ELISA. There was no significant difference in pre-operative or post-operative day one serum IL-6 concentration between patients who had an uneventful recovery and those who developed SIRS. However, on post-operative day two there was significantly more IL-6 ($p<0.05$) in serum from patients who develop SIRS (mean IL-6 concentration 122pg/ml) compared to those who did not (mean IL-6 concentration 92pg/ml) (Figure 4.4). In patients who developed SIRS, IL-6 concentration was significantly greater on post-operative day two compared to pre-operatively (pre-op 84pg/ml vs Day two 122pg/ml; $p<0.004$). This finding is in keeping with the stimulated PBMC data presented in Figure 4.2 as increased IL-6 production seen in PBMCs is also seen in serum on day two.

There were no pre-operative, post-operative day one or day two differences in serum TNF-α production between patients who had an uneventful recovery and those who developed SIRS (figure 4.4). In addition there was no significant difference in IL-10 production between the groups pre-operatively, and on days one and two post-operatively (figure 4.4). Interestingly, in both groups of patients, IL-10 concentration was significantly lower on day two compared to pre-operatively (No SIRS pre-op 194pg/ml vs No SIRS Day two 129pg/ml; SIRS pre-op 211pg/ml vs SIRS Day Two 151pg/ml) ($p<0.02$). The post-operative change in serum IL-10 is in keeping with the stimulated PBMC data presented in Figure 4.3 as reduced IL-10 production in PBMCs was also seen on day two.

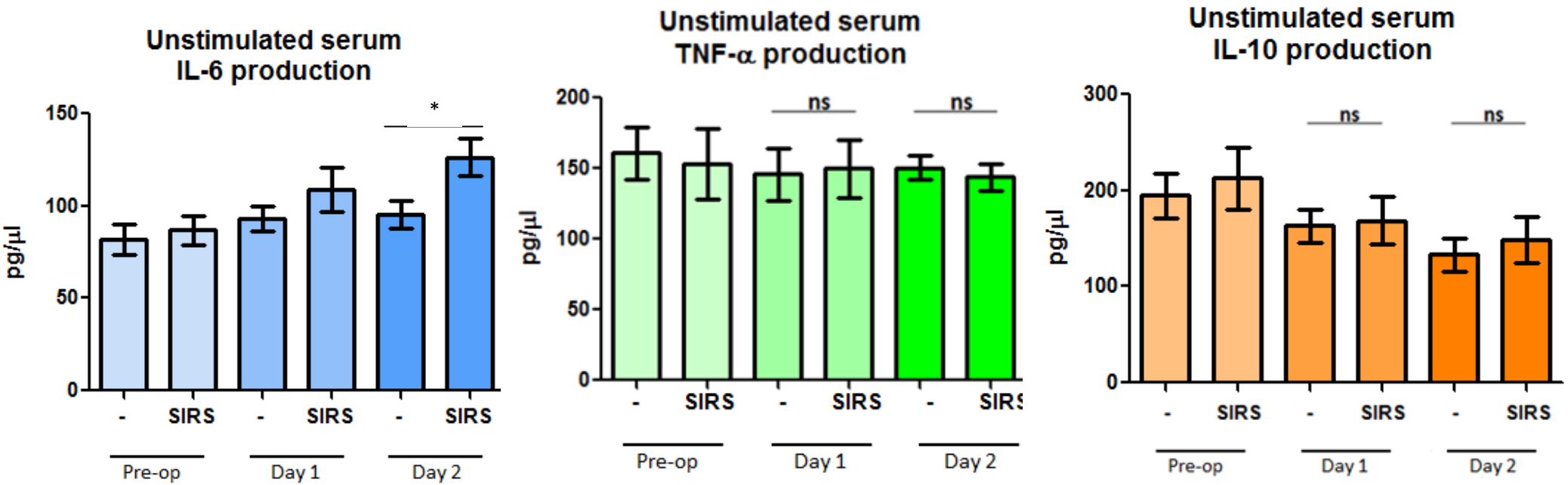


Figure 4.4. IL-6 (blue), TNF-α (green) and IL-10 (orange) production pre-operatively (pre-op), on day one (Day 1) and day two (Day 2) in patients who have an uneventful recovery (-) and patients who develop post-operative SIRS (SIRS).

4.4 Discussion

The key findings presented in this chapter are that patients who go on to develop SIRS can be distinguished from those who do not according to IL-6 production – measured directly in the serum or indirectly by ex vivo TLR-stimulated monocytes.

There was significantly greater IL-6 production in response to LPS and flagellin stimulation in patients who develop SIRS on post-operative day two. Further to this finding, there was also greater serum IL-6 concentration in patients who develop SIRS on day two. This change was a median of 4 days before patients were diagnosed with SIRS using routine clinical and biochemical parameters. There was no significant difference in TNF-α or IL-10 production in response to TLR agonists or in serum TNF-α or IL-10 in the early post-operative period. These data indicate that ex vivo agonist stimulation of PBMCs from patients who develop SIRS drives IL-6 production.

Serum IL-6 has been shown to be elevated in patients who develop sepsis following major abdominal surgery as early as post-operative day one (8) (10). These studies were completed over a decade ago and reported a rise in IL-6 on day 1 post-operatively in patients who had undergone abdominal surgery and developed sepsis. The data in this chapter in patients who develop SIRS are consistent with this. In addition, I have shown that TLR-stimulated PBMCs produce more IL-6 and hypothesise that the raised serum IL-6 is at least in part produced by a PBMC response to bacterial fragments.

The results presented in this thesis have also shown the importance of LPS (TLR4) and flagellin (TLR5) stimulated IL-6 production in PBMCs in addition to post-operative serum IL-6 concentration. This suggests a possible mechanism for IL-6 production in these patients and indicates that activation of TLR4 and TLR5 may play an important role in the increased production of IL-6 and may form a possible biomarker for post-operative SIRS. In addition,

previous studies have identified that immune responses to trauma differ significantly between men and women (158). Wichmann et al identified increased IL-6 production and reduced suppression of B and T-lymphocyte activity in women compared to men following major abdominal surgery. However, they did not evaluate changes in innate immune function. It has also been demonstrated that pro-inflammatory cytokine production from LPS stimulated monocytes was significantly greater in men than women. This study investigated stimulated whole blood, therefore cytokine production cannot be specifically attributed to monocytes. Another perceived limitation in this study design is that the sexes were not well matched (M:F 7:1).

The role of TNF-α in post-operative SIRS remains unknown. This thesis identified no difference in LPS and flagellin-stimulated TNF-α production or serum TNF-α concentration between patients who develop SIRS and those who have an uneventful recovery. A study from Japan evaluated the efficacy of Sivelestat (a selective neutrophil elastase inhibitor) in reducing SIRS following oesophagectomy. They identified no difference in TNF-α production between patients who develop SIRS and those who do not in 43 consecutive patients who underwent oesophagectomy (176). However, only one patient in the Sivelestat group developed SIRS so it is not possible to draw definite conclusions. Again, this group only assessed TNF-α concentration in patient serum and were unable to comment on a possible mechanism behind changes in cytokine production. A study that evaluated changes in serum cytokine production in 40 patients undergoing complex major abdominal surgery found elevated concentrations of TNF-α and IL-10 following surgery (16). This group showed that serum IL-6 concentration was elevated before serum TNF-α rose, which was increased on post-operative day one. As described later in this chapter, IL-6 has been shown to inhibit TNF-α production in both ex-vivo and in-vivo studies (177) and TNF-α has previously been shown to increase before IL-6 (1). The findings in this chapter are consistent with another study in patients following major vascular (178) surgery which showed that in 14 patients undergoing abdominal aortic aneurysm (AAA) repair, IL-6 production was significantly increased in patients who developed post-operative complications such as SIRS and sepsis. Although differences were seen in IL-6, there was no significant difference in TNF-α production in these patients (178). In addition to plasma cytokine concentrations,

Ziegenfuss et al. stimulated whole blood with high dose LPS (1μg/ml) and isolated PBMCs using a Ficoll© gradient to evaluate TNF-α production in addition to serum TNF-α concentration. These data suggests that the full understanding of the role of TNF-α in post-operative SIRS remains to be elucidated.

Much like TNF-α, the role of IL-10 in post-operative SIRS is not fully understood. In a study of 51 patients undergoing minor, moderate (including colectomy and gastrectomy) and major surgery (Ivor Lewis oesophagectomy), there was no difference in serum IL-10 concentration post-operatively between major and minor cases (179). The findings presented in this chapter support those published by Kashiwabara et al. However, in contrast to this, Dimopoulou et al (16) identified that serum IL-10 concentrations were elevated immediately after major surgery. Mokart et al found that unstimulated serum IL-10 concentration was elevated on post-operative day one in patients who go on to develop sepsis following abdominal surgery (8). A study using electrophoretic mobility shift assays showed that IL-10 inhibits nuclear localisation of NF-κB, which would in turn reduce inflammatory cytokine production (180). The reduction in LPS and flagellin stimulated IL-10 production on post-operative day two at the same time as IL-6 production and NF-κB phosphorylation (see chapter 6) increased in patients who develop SIRS, is in keeping with this.

These data indicate that increased IL-6 may play an important role in the development of post-operative SIRS. Although no difference was seen in TNF-α or IL-10 production between patients who develop SIRS and those with an uneventful recovery, the importance of other cytokines in post-operative SIRS cannot be understated until further time points and cytokines have been evaluated. It is likely that IL-6, TNF-α and IL-10 productions have different kinetics following surgery and peak at different times. Another possible explanation for this phenomenon in this series of experiments is that IL-6 production may inhibit TNF-α production following 24 hour LPS and flagellin stimulation. Cultured human peripheral monocytes treated with IL-6 were shown to reduce TNF-α production (177). The same group also demonstrated that LPS-induced TNF-α production in mice was decreased following pre-treatment with IL-6. As TNF-α has been shown to induce IL-6 production (181),

it is possible that IL-6 acts as a negative feedback mechanism to prevent overproduction of TNF-α and other inflammatory cytokines. Given that TNF-α levels peak before IL-6 levels following TLR agonist stimulation, it is possible that the 24 hour stimulation used in this thesis saw IL-6 levels reach the concentration required to inhibit and reduce TNF-α production. In addition to this, IL-10 has been shown to inhibit IL-6 production (182) but in patients who develop post-operative SIRS, there was a trend towards a reduction in LPS and flagellin stimulated IL-10 production following surgery. This suggests that IL-10 may be one of the key mediators in preventing over production of IL-6. This negative feedback loop of TNF-α production may account for the fact that no differences were seen between patients who had an uneventful recovery and those who developed SIRS. With this in mind, it might be interesting to study the kinetics of the experiments outlined in this chapter and in subsequent chapters to better understand the mechanism by which IL-6 but not TNF or IL-10 production distinguishes patients who develop SIRS from those who do not.

ELISA was chosen to evaluate IL-6, TNF-α and IL-10 production because robust, commercially available ELISA kits are available and inexpensive. It is well known that these 3 cytokines play an integral role in the evolution of SIRS which is why they were studied. However, the cytokine dysregulation which characterises SIRS is complex, and it is likely that IL-6 will not be the only cytokine that can distinguish between those who develop post-operative SIRS and those who do not. There are other experimental modalities that could have been used to evaluate cytokine production. It was tempting to use multiplex technology to study a large number of markers (e.g. up to 30 using Luminex technology). Luminex assays are an evolution of the principle of ELISA. However, given that each patient had 9 experimental conditions (unstimulated, LPS and flagellin stimulated on 3 different days) and would need to be run in duplicate, each plate could analyse only four patients and costs of this technique were prohibitive at this stage. However, even this sort of approach introduces bias, because it is only known markers that are studied in these platforms. Proteomic and transcriptomic approaches could minimise this bias, but given the costs of these platforms and the problem with kinetics described above, this would represent an unwieldy set of experiments. ELISPOT is a modified version of ELISA. The major difference is that ELISPOT allows quantification of cytokine production at the single cell level. This is very useful when

looking at rare cell populations but was not required when looking at LPS and flagellin-stimulated cytokine production in PBMCs.

Given that IL-6 production was dysregulated following LPS and flagellin stimulation in patients who develop SIRS, it is possible that changes at the receptor level are driving the changes seen in cytokine production. To test this hypothesis, I decided to evaluate TLR4 and TLR5 monocyte cell surface expression. This was done to determine whether the increased IL-6 production was due to changes in receptor expression.

5. Evaluating changes in TLR4 and TLR5 expression on different monocyte subpopulations in the peri-operative period

5.1 Introduction

The ex-vivo monocyte functional data in the previous chapter identified an early post-operative increase IL-6 production in response to LPS (TLR4) and flagellin (TLR5) agonists in patients who go on to develop SIRS. Given the enhanced ex-vivo functional response in these patients, I evaluated TLR4 and TLR5 cell surface expression on monocytes to test the hypothesis that altered receptor expression was driving increased cytokine production in patients that develop post-operative SIRS.

Flow cytometry is an excellent technique for rapid analysis of the specific cell type (e.g. monocytes) within a heterogenous cell population (e.g. PBMCs). Antibodies allow specific binding to proteins of interest without disruption of cell architecture. Flow cytometry is also validated for use in assessing TLR expression on various cell types with commercially antibodies available. Another benefit of flow cytometry is that when a protocol has been formulated and optimised, it is easily repeatable which facilitates high throughput processing of samples. It also allows the measurement of multiple parameters within a single experiment, thereby reducing intra-experiment variability. Like all experimental techniques, flow cytometry has potential drawbacks. Fluorophore spill over can be a significant weakness of flow cytometry. However, this is corrected for using compensation beads. A common criticism of flow cytometry is the lack of standardisation in assay and instrument set up. Accurate gating strategies to identify cell populations require expertise and experience in flow cytometry. Cost (incurred through antibody use and flow cytometer time) is a significant drawback of using this technique and there is, as yet, also no standard of data reporting in flow cytometry (like the MIQE guidelines for qPCR).

The data in this chapter demonstrates that on post-operative day one, there was a significant increase in TLR4 and TLR5 expression on unstimulated monocytes in patients who go on to develop SIRS compared to those that had an uneventful recovery. This increase in TLR4 and TLR5 expression was more significant on post-operative day 2.

5.2 Identifying the monocyte ($CD14^+$) population using flow cytometry

Flow cytometry was used to evaluate TLR4 and TLR5 cell surface expression on monocytes. A broad initial gate was drawn to exclude dead cells and debris (Figure 5.1). A flow cytometry plot of CD14 against AmCyan-A (an empty fluorophore channel) was used for identification of the $CD14^+$ population (Figure 5.1). The $CD14^+$ cells were then isolated and TLR4 was plotted on the Y axis and TLR5 was plotted on the X axis (Figure 5.1). The Median Fluorescence Intensity (MFI) of TLR4 and TLR5 expression on $CD14^+$ cells was calculated by the software programme. Plotting TLR4 and TLR5 allowed the MFI of both to be recorded on the same data panel, which increased the ease of data collection. The MFI of TLR4 and TLR5 was used to compare TLR4 and TLR5 expression on monocytes between patients who had an uneventful recovery and those who developed SIRS.

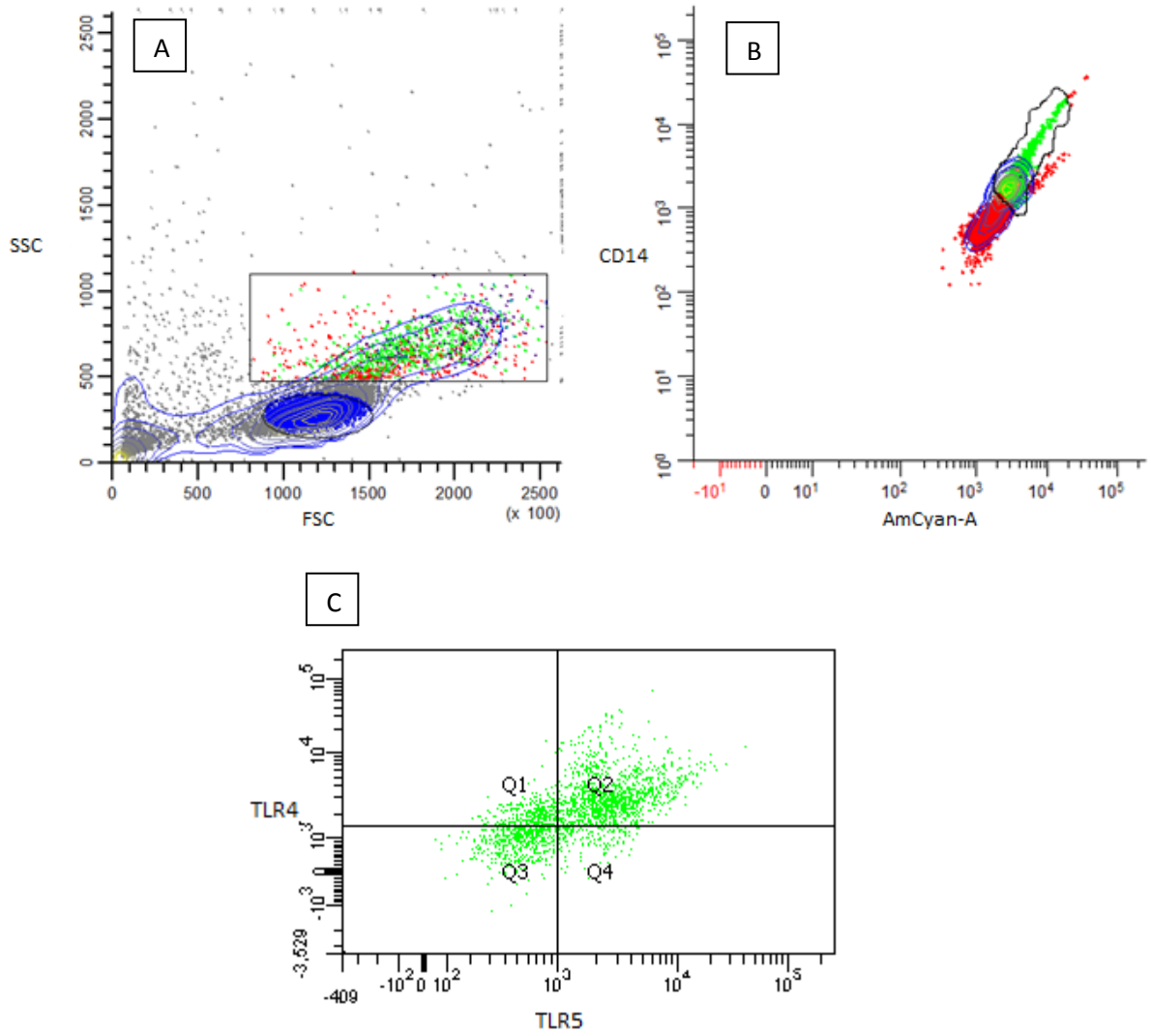


Figure 5.1. A) Gating of probable monocytes based on morphology. (B) $CD14^{+}$ cells identified in a plot of CD14 MFI versus an empty fluorophore channel (Am-Cyan-A) (C) Flow cytometry plot of TLR4 versus TLR5.

5.3 Comparison of TLR4 and TLR5 expression on monocytes from patients with an uneventful recovery and patients who go on to develop post-operative SIRS

5.3.1 TLR4 and TLR5 expression on unstimulated monocytes

Pre-operatively, there was no significant difference in TLR4 or TLR5 expression on monocytes between patients who developed post-operative SIRS and those with an uneventful recovery (Figure 5.2). However, on post-operative days one (i.e. within 24 hours of surgery) and two, TLR4 (No SIRS Day One MFI 1683; SIRS Day One MFI 3435; $p<0.0001$) (No SIRS Day Two MFI 2158; SIRS Day Two MFI 4723; $p<0.0001$) and TLR5 (No SIRS Day One MFI 1328; SIRS Day One MFI 3323; $p<0.0001$) (No SIRS Day Two MFI 2186; SIRS Day Two MFI 5358 MFI; $p<0.0001$) on unstimulated monocytes in patients who go on to develop SIRS were significantly greater compared to patients who had an uneventful recovery (Figure 5.2). The magnitude of these differences was greater on day two than on day one.

I also examined the changes in TLR expression over time in each patient. In patients who did not develop SIRS, there was no significant change in TLR4 and TLR5 expression on monocytes over the peri-operative period (Figure 5.3). However, in the SIRS group, there was a significant sequential increase in TLR4 ($p<0.0002$ one way ANOVA) and TLR5 ($p<0.0001$ one way ANOVA) expression on post-operative days one and two (Figure 5.3).

Taken together, these findings demonstrate that increased TLR4 and TLR5 expression on unstimulated monocytes on days one and two after major surgery identify patients that go

on to develop SIRS. These changes occur significantly before the majority of patients were diagnosed with SIRS.

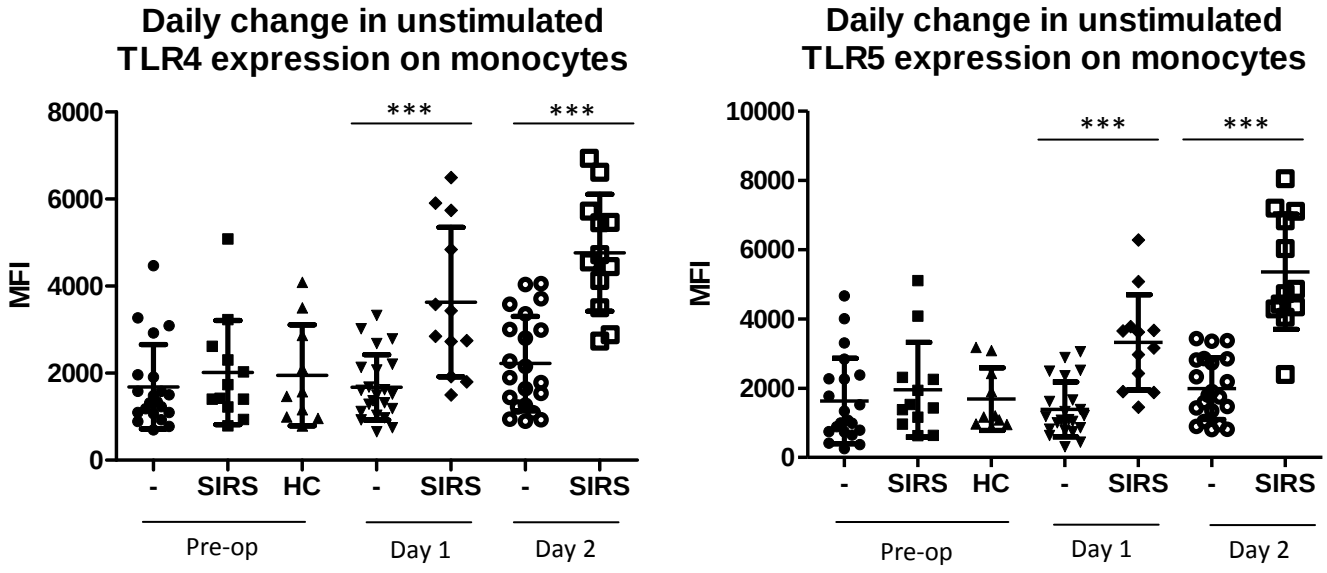


Figure 5.2. The comparison of pre-operative (pre-op) and early post-operative (day one and day two) unstimulated TLR4 and TLR5 expression in patients that develop SIRS (SIRS) and those who have no complications (-). TLR4 and TLR5 expression in healthy controls (HC) is also shown.

As shown in Figure 5.2, there is no significant change in monocyte TLR4 and TLR5 expression in patients who had an uneventful recovery. This was not the case in patients who develop SIRS, where a steady increase in TLR4 and TLR5 expression was seen. There was a strong positive correlation between TLR4 expression and LPS-stimulated IL-6 production (Figure 5.4). This was also the case between TLR5 expression and flagellin-stimulated IL-6 production (Figure 5.5). This suggests an association between increased TLR4 and TLR5 expression and greater IL-6 production following stimulation with the relevant agonist. As there was no significant difference in TNF-α or IL-10 production between the groups, I did not analyse for a correlation between TLR expression and TNF-α or IL-10 production.

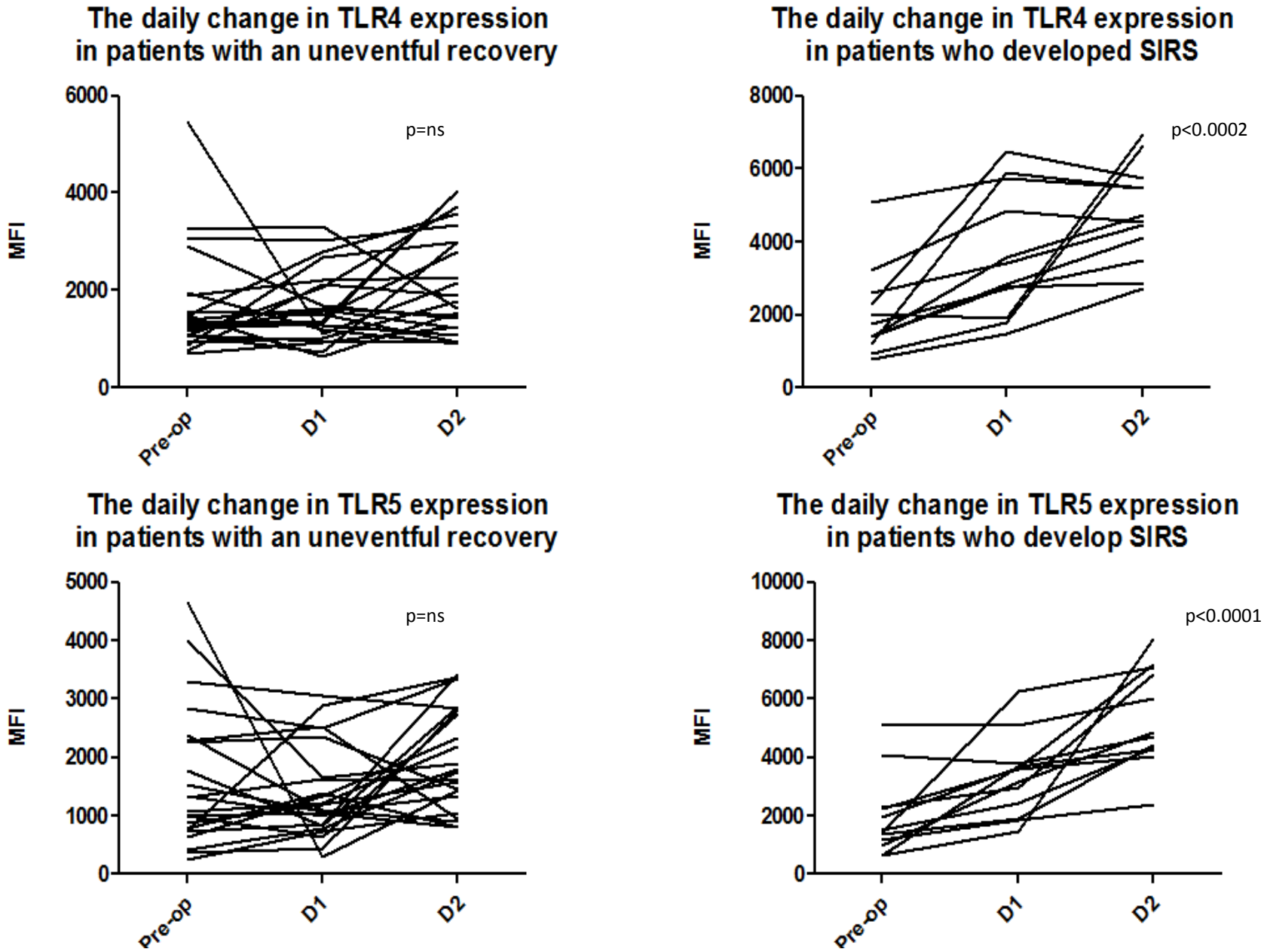


Figure 5.3. The change in TLR4 and TLR5 expression over time in patients who have an uneventful recovery and those that develop SIRS. (This data is the same as that plotted in figure 3.3. It has been plotted as a line graph to illustrate the changes in TLR expression between the groups.)

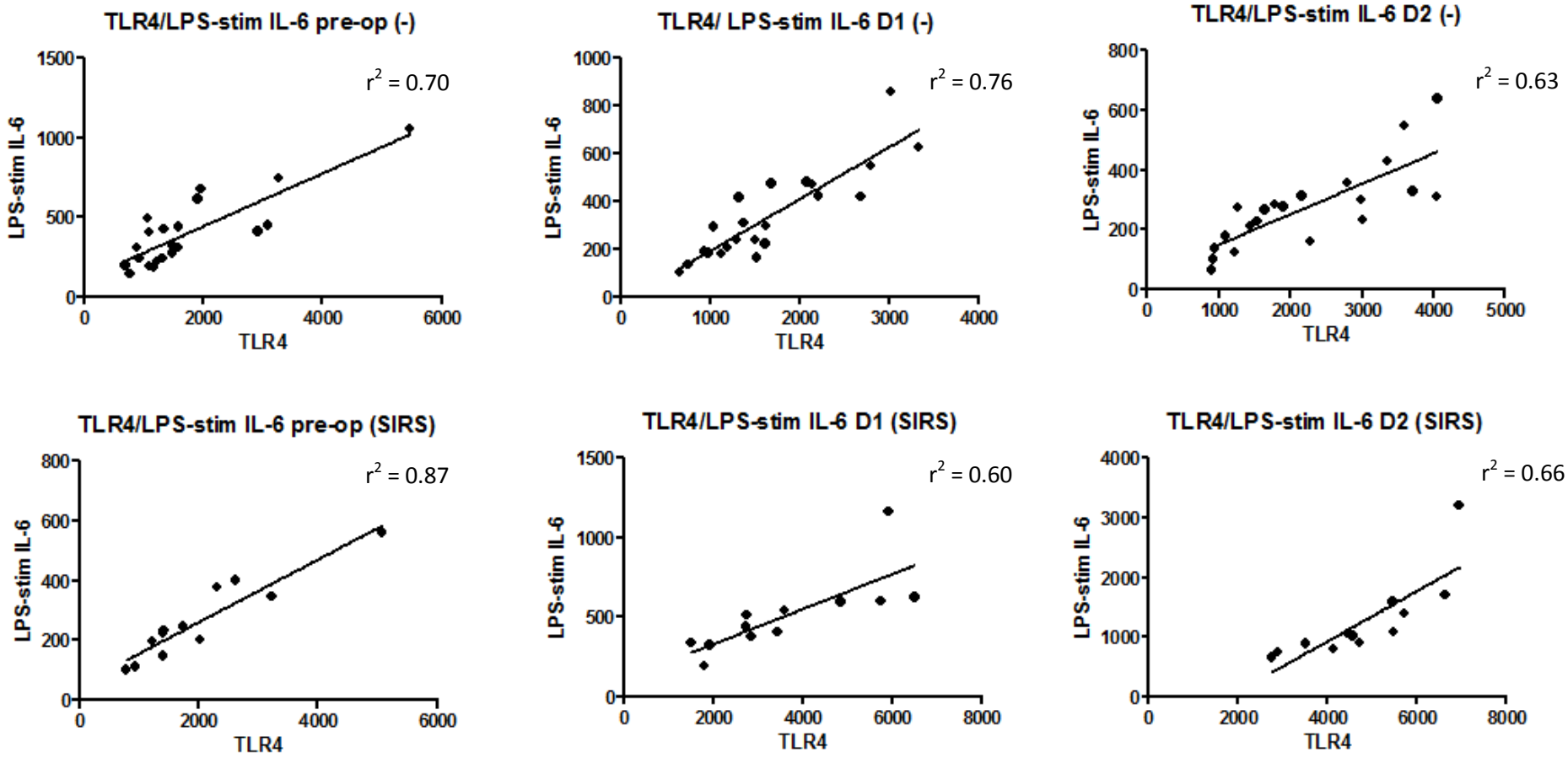


Figure 5.4. The positive correlation between the pre-operative (pre-op), post-operative day one (D1) and day two (D2) TLR4 expression and LPS-stimulated IL-6 production in patients who have an uneventful recovery (-) and those who develop SIRS. The r^2 coefficient is presented with each graph.

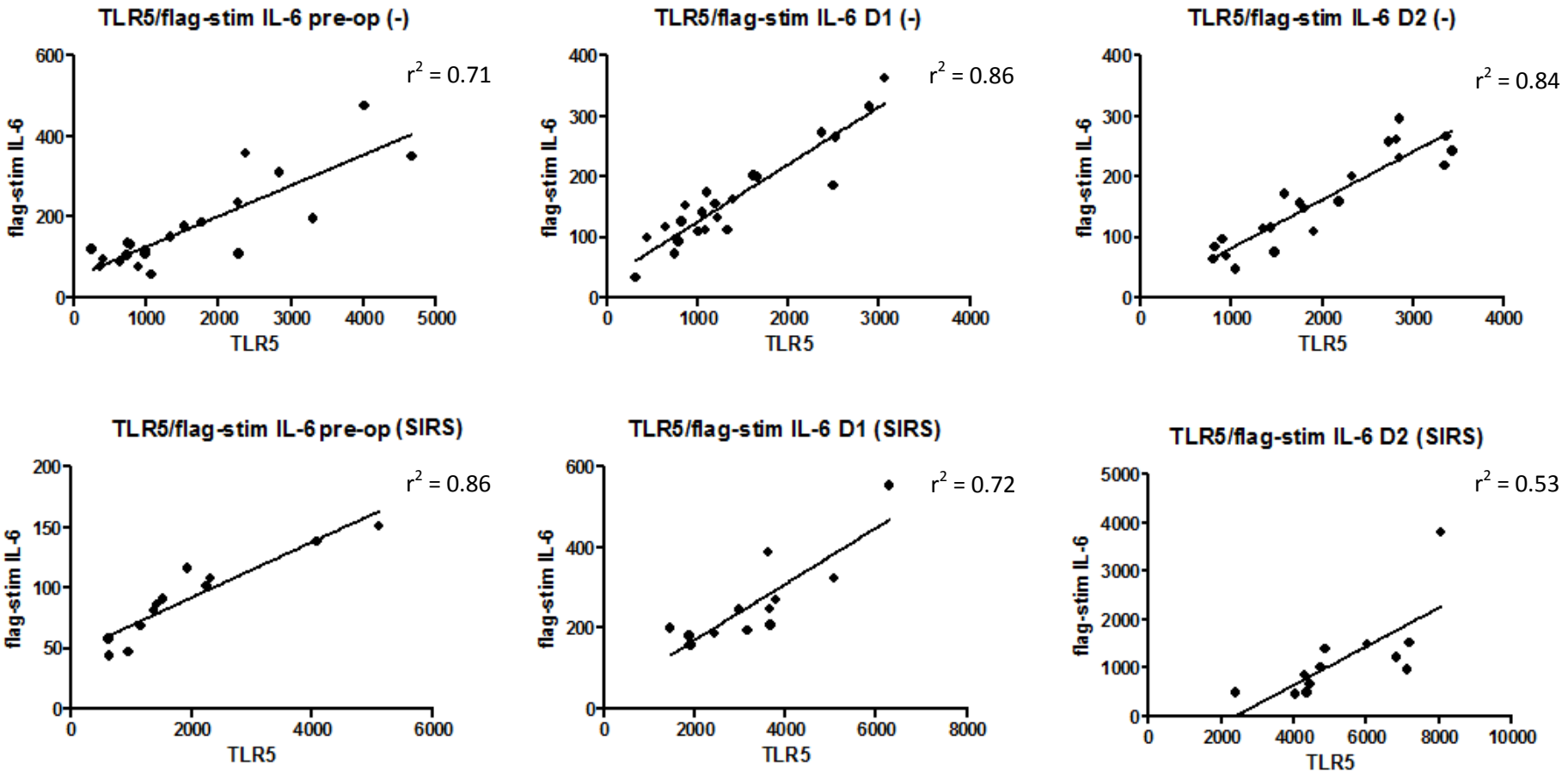


Figure 5.5. The positive correlation between the pre-operative (pre-op), post-operative day one (D1) and day two (D2) TLR5 expression and LPS-stimulated IL-6 production in patients who have an uneventful recovery (-) and those who develop SIRS. The r^2 coefficient is presented with each graph.

5.3.2 The effect of patient serum on unstimulated healthy control monocyte TLR4 and TLR5 expression and cytokine production

As identified above, monocytes from patients who develop post-operative SIRS have increased TLR4 and TLR5 expression in the early post-operative period. Given that LPS and flagellin stimulated PBMC IL-6 production and serum IL-6 concentration was significantly increased in patients who developed SIRS on day two, it was still unclear whether serum contained a circulating factor that could influence monocytes, leading to the changes seen in IL-6 production and TLR expression. To evaluate the role of serum on TLR expression and cytokine production, I incubated patient serum with PBMCs from healthy controls. Cells were pooled from healthy volunteers to obtain a consistent test population. Healthy PBMCs were incubated with patient serum and stimulated with LPS or flagellin. The cells were then incubated for 24 hours. TLR4 and TLR5 expression on monocytes was analysed using flow cytometry. Supernatants were stored and analysed using a commercially available ELISA. I tested the hypothesis that serum-conditioning led to changes in TLR4 and TLR5 expression on healthy PBMCs.

To identify whether the process of pooling healthy PBMCs significantly changed TLR4 and TLR5 expression on pooled healthy monocytes compared to a single healthy control, the same flow cytometry protocol was performed. This identified that there was no significant difference in TLR expression when comparing pooled PBMCs to individual healthy controls (Figure 5.6). Following this confirmation, pooled PBMCs and PBMCs from a healthy control were treated with healthy control serum to identify if the process of pooling different PBMCs significantly changed TLR4 and TLR5 expression on pooled monocytes compared to individual healthy controls (Figure 5.7). This was not shown to be the case as there was no significant difference in TLR expression between the treated pooled PBMCs and the individual healthy controls (Figure 5.7).

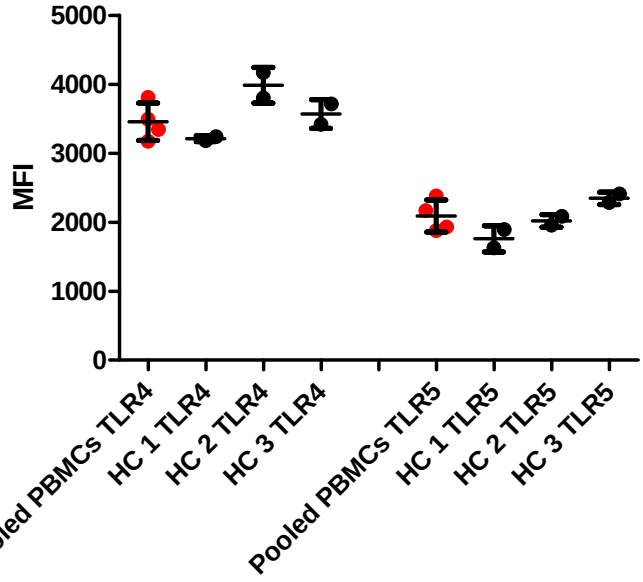


Figure 5.6. Unstimulated monocyte TLR4 and TLR5 cell surface expression of pooled healthy PBMCs in comparison to healthy control PBMCs (HC 1, HC 2, HC 3).

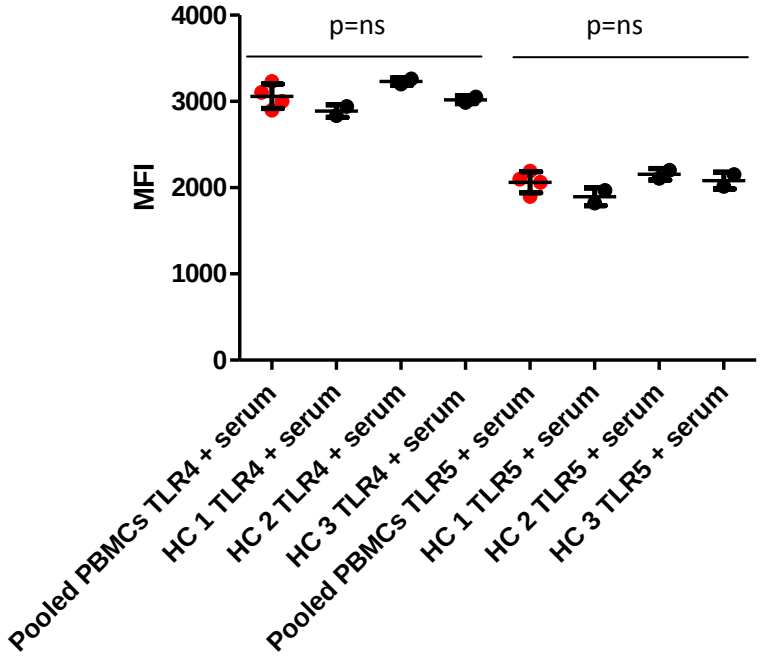


Figure 5.7. Unstimulated monocyte TLR4 and TLR5 cell surface expression of pooled healthy PBMCs treated with healthy control serum in comparison to healthy control PBMCs treated with healthy control serum (HC 1, HC 2, HC 3).

The supernatants were used to determine if there was a significant increase in IL-6 production (Figures 5.8 and 5.9). There was no significant increase in IL-6 production in pooled healthy PBMCs alone or pooled healthy PBMCs treated with healthy serum compared to healthy control PBMCs. Therefore, pooled healthy control PBMCs were used to evaluate the role of patient serum in the post-operative inflammatory response.

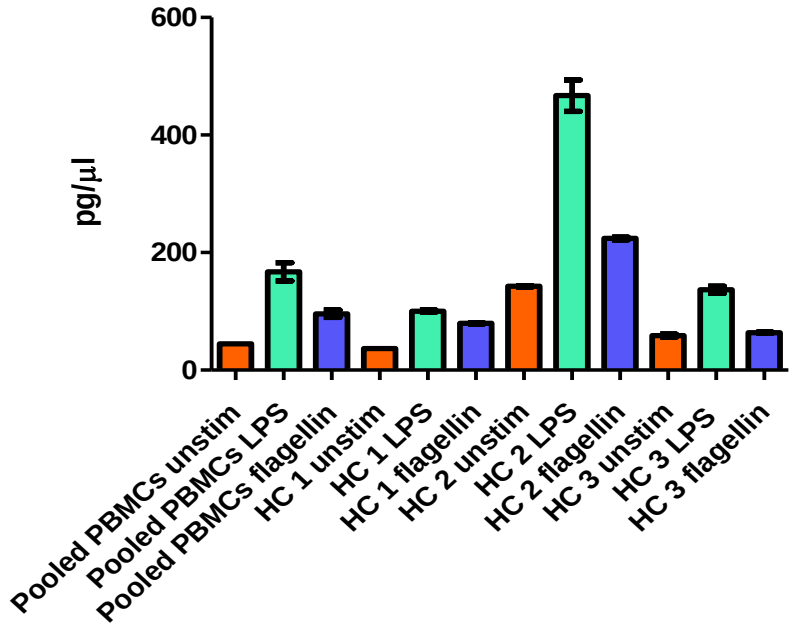


Figure 5.8. IL-6 production of unstimulated pooled healthy PBMCs in comparison to healthy control samples (HC 1, HC 2, HC 3).

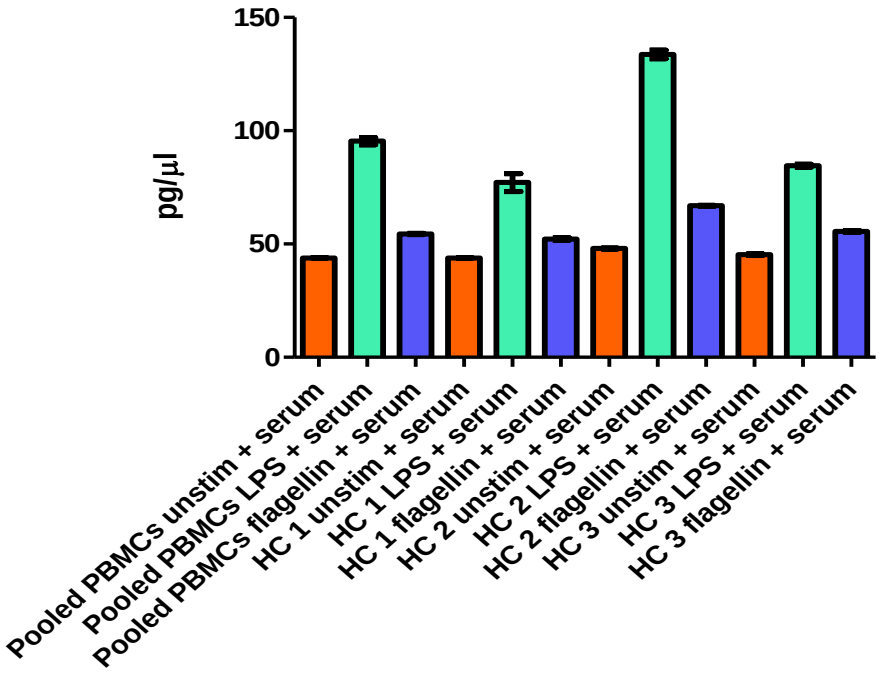


Figure 5.9. IL-6 production of serum treated unstimulated pooled healthy PBMCs in comparison to serum treated healthy control samples (HC 1, HC 2, HC 3).

Following treatment of unstimulated healthy control pooled PBMCs with pre-operative patient serum; there was no significant difference in TLR4 or TLR5 expression on pooled monocytes treated with serum from patients with an uneventful recovery and those treated with serum from patients who develop SIRS (Figure 5.10). There was no significant difference in TLR4 or TLR5 expression on pooled monocytes treated with day one or day two serum from patients who had an uneventful recovery and those who developed SIRS. (Figure 5.10). In addition, there was no significant difference in TLR4 or TLR5 expression on unstimulated serum-treated pooled monocytes when comparing pre-operative and day one values. Taken together, these findings show that the changes in TLR4 and TLR5 expression could not be induced by a stable serum factor. Rather, the changes seen in TLR expression and IL-6 production are likely to be intrinsic to the monocyte itself.

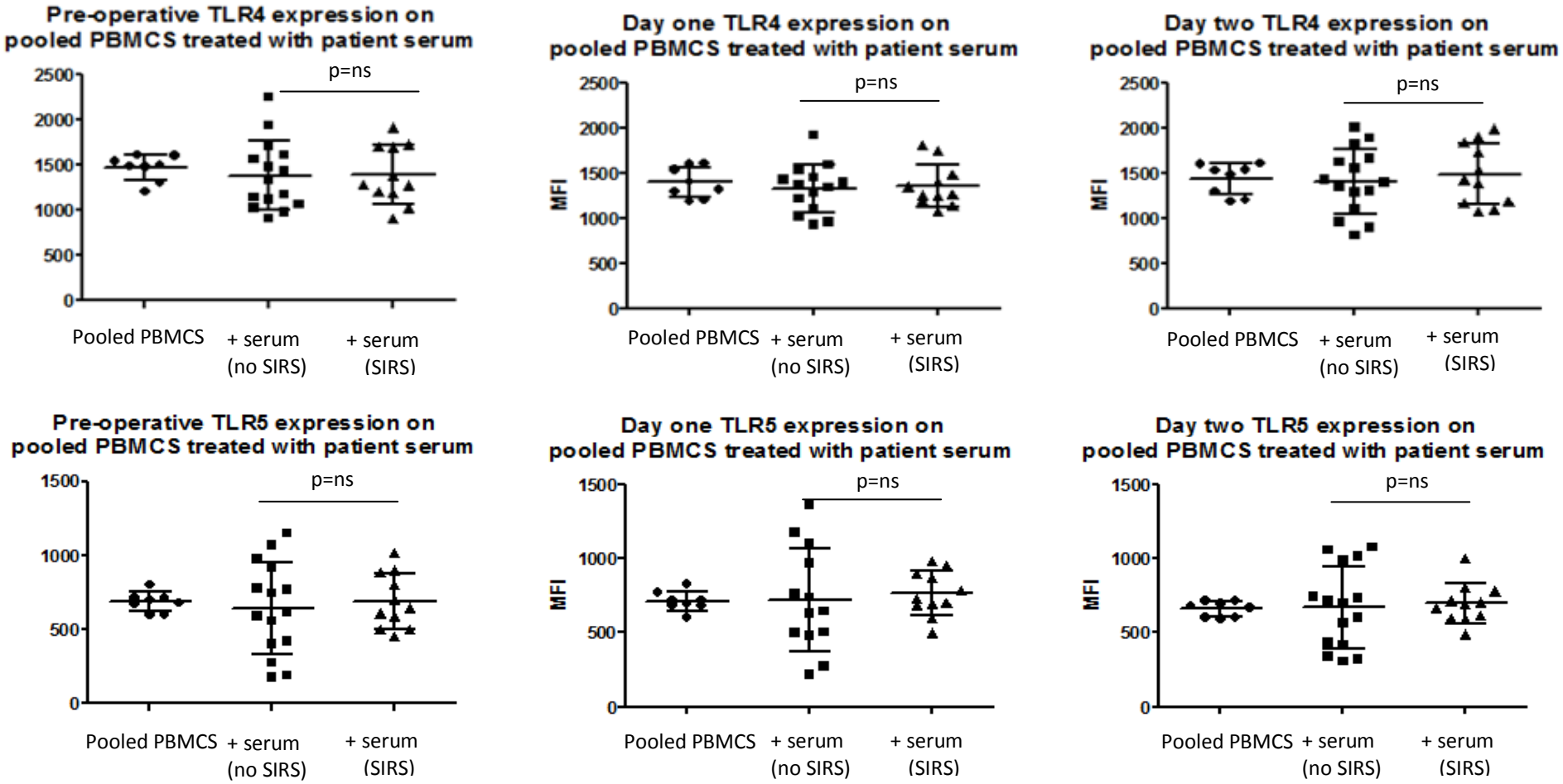


Figure 5.10. Pre-operative, day one and day two TLR4 and TLR5 expression on pooled healthy control PBMCs. TLR expression was evaluated on pooled PBMCs (n=7), pooled PBMCs treated with serum from patients who had an uneventful recovery (+ serum (no SIRS) (n=15) and pooled PBMCs treated with serum from patients who developed SIRS (+ serum (SIRS)) (n=11).

The use of serum conditioned media from patients did not recapitulate the cytokine findings seen in Chapter 4 or the changes in TLR expression shown in this chapter on PBMCs from healthy controls. Following LPS stimulation, there was no significant difference in IL-6, TNF-α or IL-10 production (Figure 5.11) between patients who develop SIRS and those with an uneventful recovery. There was no significant difference in production of the three different cytokines pre-operatively following LPS stimulation. After incubating post-operative day one and day two serum from patients with healthy PBMCs and stimulating with LPS, there was again no significant difference in IL-6, TNF-α or IL-10 production between patients who develop SIRS and those who have an uneventful recovery (Figure 5.11).

There was also no significant difference in IL-6, TNF-α or IL-10 between patients who developed SIRS and those who had an uneventful recovery when pre-operative serum was incubated with healthy PBMCs and stimulated with flagellin (Figure 5.12). Following incubation of day one and day two serum with pooled PBMCs and subsequent flagellin stimulation, there was no significant difference in IL-6, TNF-α or IL-10 production between patients who develop SIRS and those who have an uneventful recovery (Figure 5.12).

Interestingly, production of IL-6, TNF-α and IL-10 was lower following serum-conditioned media incubation of healthy PBMCs than in stimulated patient PBMCs alone, although this did not reach statistical significance. This indicates that serum-conditioned media attenuates both pro and anti-inflammatory stimulated cytokine production. These results indicate that a readily detectable circulating factor is not present in serum that leads to increased LPS and flagellin stimulated IL-6 production in patients who develop SIRS. However we cannot exclude the possibility that a labile compound is produced and is responsible for these changes. This suggests that the increase in IL-6 production in patients who develop SIRS is intrinsic to the PBMCs.

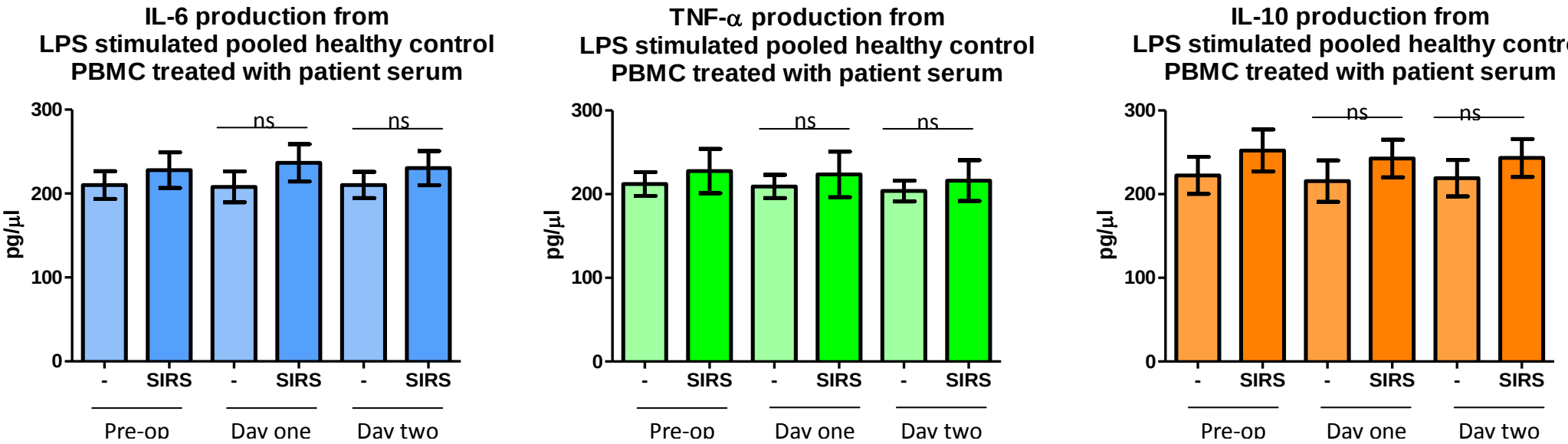


Figure 5.11. LPS stimulated IL-6 (blue), TNF-α (green) and IL-10 (orange) production in pooled PBMCs that were incubated with pre-operative (pre-op), post-operative day one (Day one) and (Day two) serum from patients who had an uneventful recovery (-) and those who developed post-operative SIRS (SIRS).

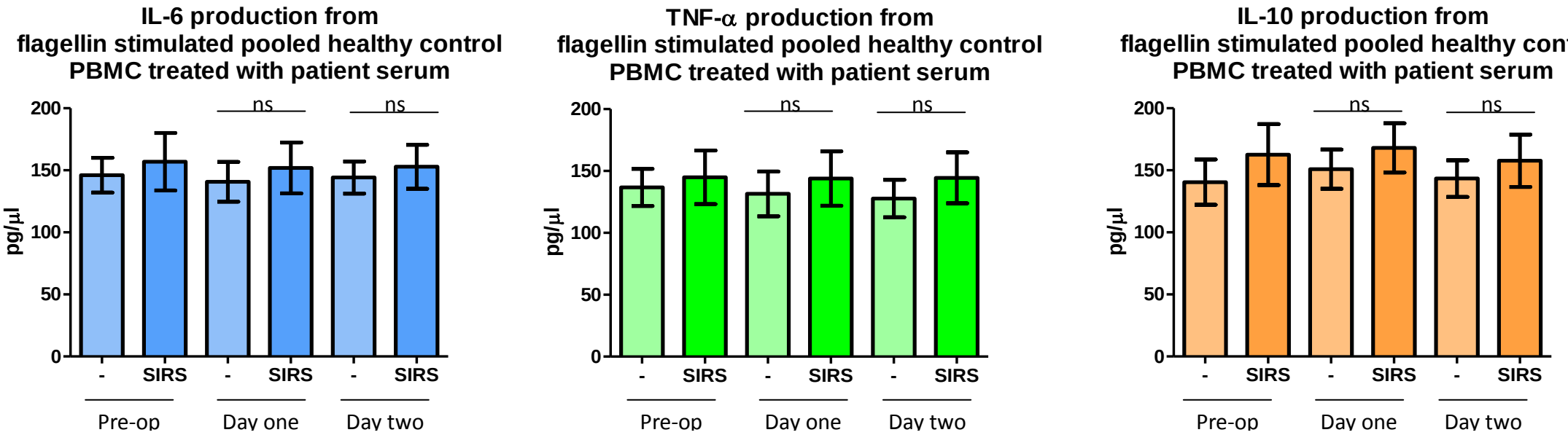


Figure 5.12. Flagellin stimulated IL-6 (blue), TNF-α (green) and IL-10 (orange) production in pooled PBMCs that were incubated with pre-operative (pre-op), post-operative day one (Day one) and (Day two) serum from patients who had an uneventful recovery (-) and those who developed post-operative SIRS (SIRS).

5.3.3 Changes in monocyte TLR4 and TLR5 expression following LPS and flagellin stimulation

Having shown differences in TLR4 and TLR5 expression in unstimulated monocytes, I investigated whether LPS and flagellin altered receptor expression on the monocyte surface and whether these induced levels of TLR4 and TLR5 were also different in patients who develop SIRS compared to those who do not. This was done on pre-operative and post-operative day one and day two samples to evaluate changes in the peri-operative period. The dose of LPS and flagellin used was the same as that used to produce IL-6, TNF-α and IL-10 production.

Pre-operatively, LPS and flagellin stimulation did not cause a significant change in monocyte TLR4 or TLR5 expression compared to unstimulated monocytes (Figure 5.13). This was the case in patients who had an uneventful recovery and those patients who developed post-operative SIRS. On post-operative day one, there was no significant difference in monocyte TLR4 and TLR5 expression following LPS and flagellin stimulation compared to unstimulated monocytes (Figure 5.14). This was the case for both patients who had an uneventful recovery and those patients who developed SIRS. On post-operative day two, in patients who went on to develop post-operative SIRS, TLR4 (Unstimulated Day Two MFI 4723; Flagellin Day Two MFI 3612; $p<0.05$) and TLR5 (Unstimulated Day Two MFI 5358; Flagellin Day Two MFI 3845; $p<0.04$) expression on unstimulated monocytes was significantly greater than TLR4 and TLR5 expression following flagellin stimulation (Figure 5.15).

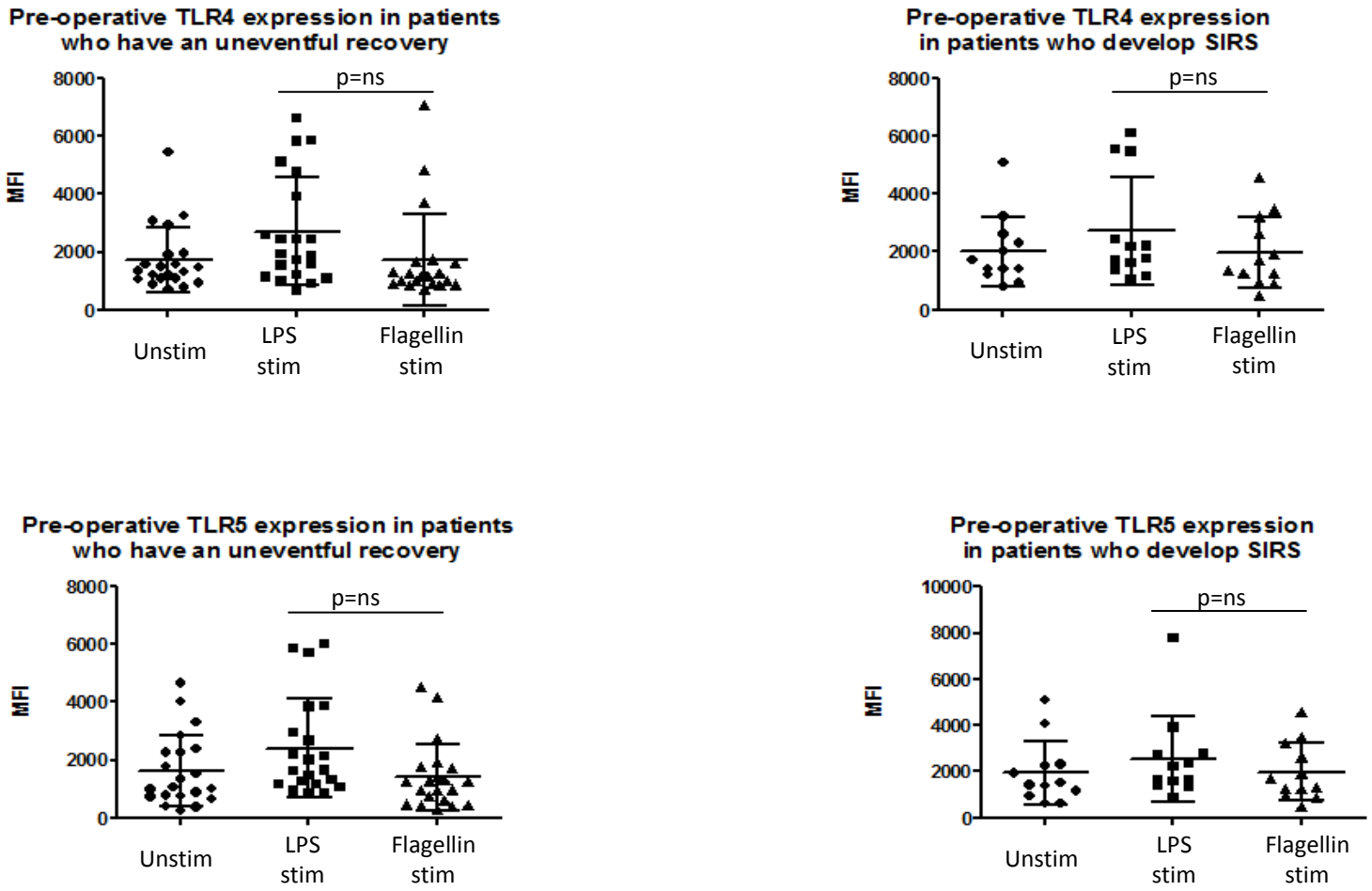
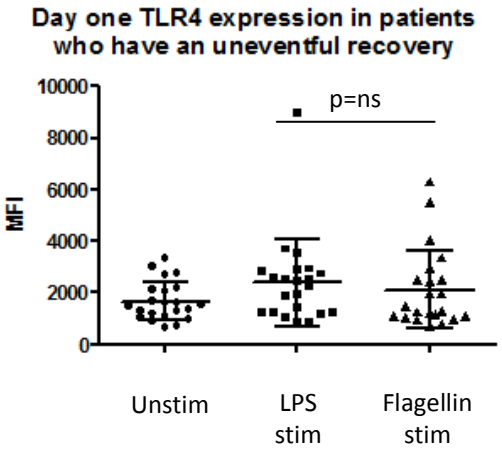
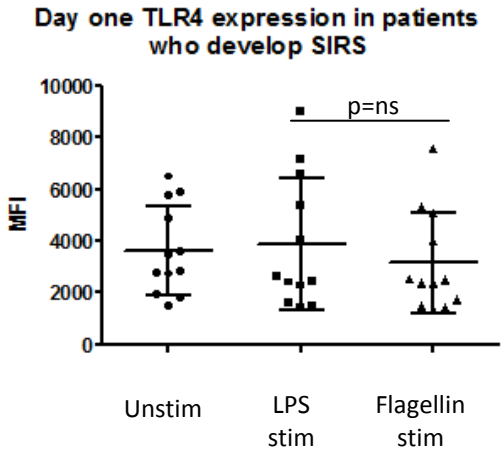
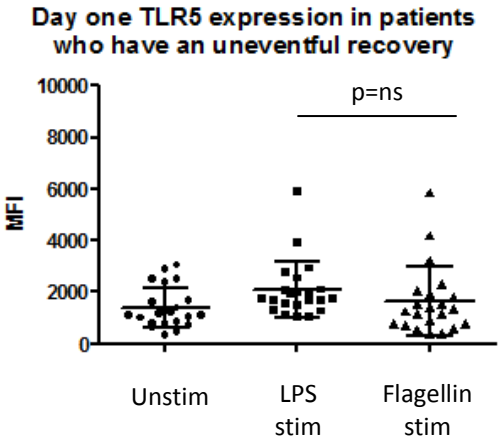


Figure 5.13. Pre-operative TLR4 and TLR5 expression on unstimulated, LPS and flagellin stimulated monocytes. There was no significant difference in TLR4 or TLR5 expression between unstimulated or stimulated monocytes.







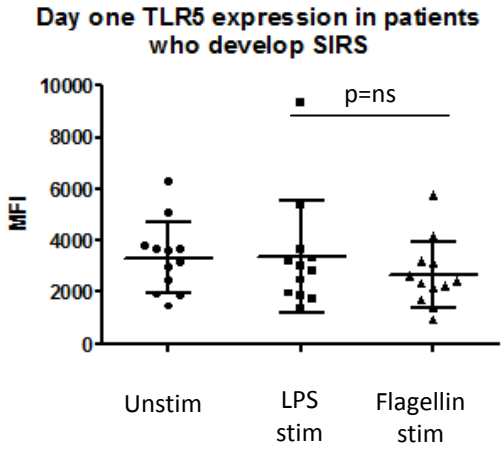


Figure 5.14. Post-operative day one TLR4 and TLR5 expression on unstimulated, LPS and flagellin stimulated monocytes. There was no significant difference in TLR4 or TLR5 expression between unstimulated or stimulated monocytes on the first day following surgery.

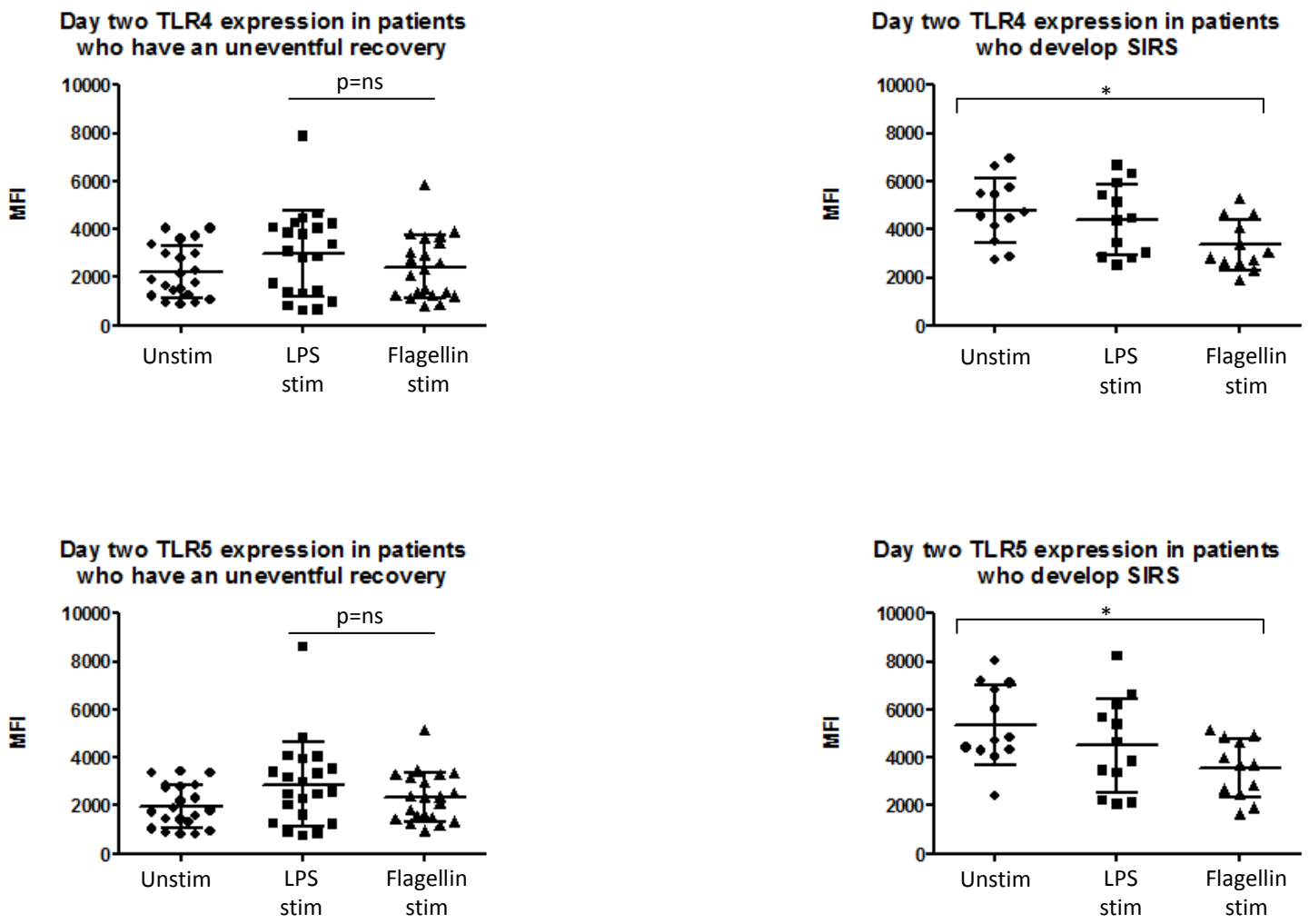


Figure 5.15. Post-operative day two TLR4 and TLR5 expression on unstimulated, LPS and flagellin stimulated monocytes. Both TLR4 ($p<0.03$) and TLR5 ($p<0.04$) expression were significantly increased on monocytes compared to flagellin-stimulated monocytes.

5.4 Identification of TLR4 and TLR5 expression on different monocyte subpopulations

Monocytes can been characterised into 3 separate subpopulations. This is determined by the intensity of CD14 and CD16 expression on the cell surface. It is now known that production of inflammatory cytokines differs between different monocyte subpopulations. Following the identification of increased global monocyte TLR4 and TLR5 expression in patients who develop SIRS, I evaluated whether this change was present in a particular monocyte subpopulation (classical ($CD14^{+}/CD16^{-}$), intermediate ($CD14^{+}/CD16^{+}$) or non-classical ($CD14^{dim}/CD16^{+}$)) or whether a global change in all monocytes was responsible for this phenomenon.

To identify different monocyte subpopulations, anti-CD16 antibody was added to the flow cytometry protocol described in Chapter 2. Following compensation to calibrate the flow cytometry software for the new antibody, gates were redrawn to identify the different monocyte subpopulations based on antibody staining (Figure 5.16). Monocytes were again initially gated for morphologically and then by antibody staining to exclude all other cells. Following this, different monocyte subpopulations were gated based upon the strength of their CD14 and CD16 staining (Figure 5.16). The data presented in this section is based upon PBMC aliquots from the patients analysed above. No new patients were analysed for monocyte subpopulation TLR expression.

5.4.1 Percentage change in classical, intermediate and non-classical monocytes before and after major HPB surgery

Pre-operatively, patients who went on to develop post-operative SIRS had a significantly lower proportion of classical monocytes (61.9% of total population; $p<0.05$) and significantly higher proportion of intermediate monocytes (29.5% of total population; $p<0.01$) among the total monocyte population than patients who had an uneventful recovery (classical monocytes 71.3%; intermediate monocytes 20.7%) (Figure 5.17). On post-operative days one and two, there was no significant difference in classical, intermediate or non-classical monocyte populations between patients who develop SIRS and those who have an uneventful recovery (Figure 5.17).

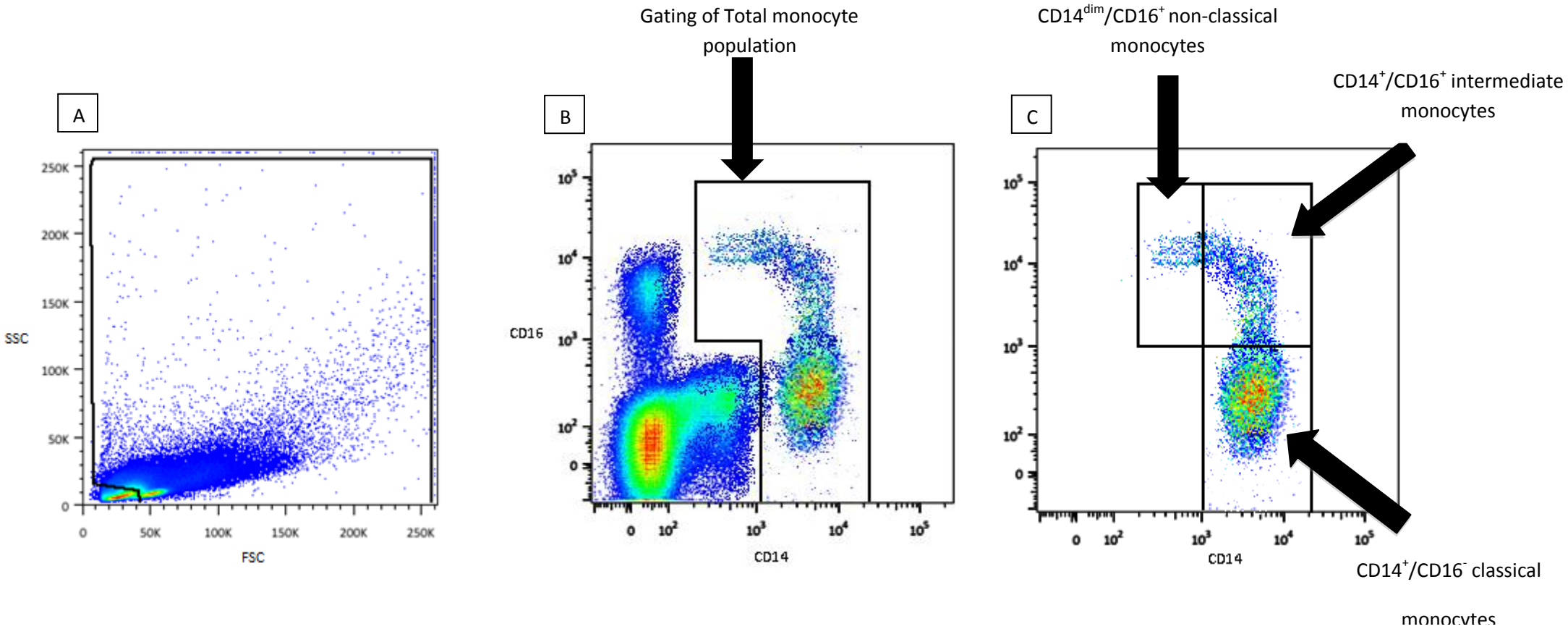


Figure 5.16. Gating different monocyte subpopulations. A large morphological gate excluded dead cells (A). The whole monocyte population was identified based upon CD14 and CD16 antibody staining (B). Classical ($CD14^+/CD16^-$), Intermediate ($CD14^+/CD16^+$) and non-classical ($CD14^+/CD16^+$) monocyte subpopulations were identified based upon CD14 and CD16 staining (C). TLR4 and TLR5 expression was then evaluated for each monocyte population.

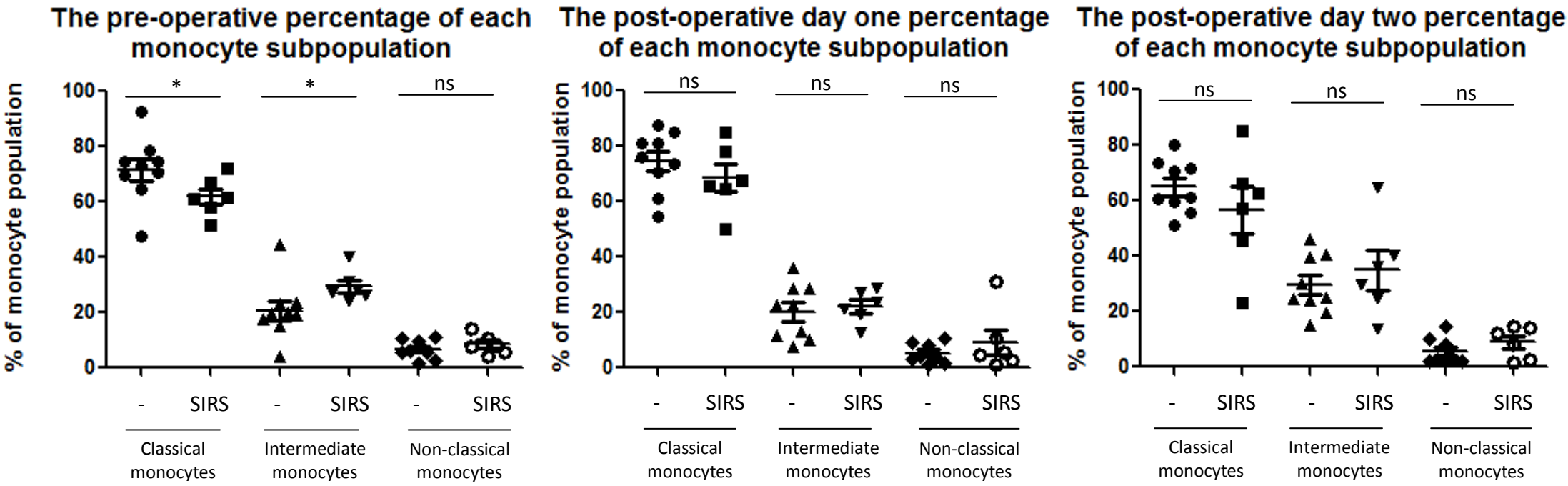


Figure 5.17. Changes in monocyte subpopulations in the peri-operative period. The percentage change in classical ($CD14^{+}/CD16^{-}$), intermediate ($CD14^{+}/CD16^{+}$) and non-classical ($CD14^{dim}/CD16^{+}$) monocytes between patients who have an uneventful recovery (-) and those who develop SIRS (SIRS) is shown

5.4.2 TLR4 and TLR5 expression on different monocyte subpopulations

There was no difference in pre-operative TLR4 or TLR5 expression on classical or intermediate monocytes between patients who had an uneventful recovery and those who developed post-operative SIRS (Figure 5.18). As expected, intermediate monocytes in both groups had significantly greater TLR4 (No SIRS classical monocytes MFI 1565 vs No SIRS intermediate monocytes MFI 7802; $p<0.0001$) (SIRS classical monocytes MFI 1764 vs SIRS intermediate monocytes MFI 8029; $p<0.0001$) and TLR5 (No SIRS classical monocytes MFI 1335 vs No SIRS intermediate monocytes MFI 3486; $p<0.0001$) (SIRS classical monocytes MFI 1939 vs SIRS intermediate monocytes MFI 3527; $p<0.0001$) expression than classical monocytes. Increased TLR4 expression on intermediate monocytes has been demonstrated previously in whole blood culture in response to sepsis-relevant antigens (183). On both post-operative days one and two, TLR4 (No SIRS Day One MFI 7855 vs SIRS Day One MFI 9931; $p<0.0004$) (No SIRS Day Two MFI 7952 vs SIRS Day Two MFI 11963; $p<0.0001$) and TLR5 (No SIRS Day One MFI 4498 vs SIRS Day One MFI 6886; $p<0.0002$) (No SIRS Day Two MFI 6025 vs SIRS Day Two MFI 8892; $p<0.0001$) expression on intermediate monocytes was significantly greater in patients who developed SIRS compared to patients who had an uneventful recovery (Figure 5.18). In patients who developed post-operative SIRS, TLR4 expression on intermediate monocytes was significantly greater on days one ($p<0.02$) and two ($p<0.0001$) than pre-operatively. This was also the case for TLR5 expression (day one; $p<0.01$, day two; $p<0.0001$). There was no significant change in intermediate monocyte TLR4 and TLR5 expression in patients who had an uneventful recovery. There was also no significant change in TLR4 and TLR5 expression on classical monocytes in the peri-operative period in either group of patients.

Taken together, these data indicate that pre-operatively, patients who develop SIRS have a larger proportion of intermediate monocytes. There is an increase in the proportion of intermediate monocytes following surgery in all patients, such that this difference is no longer apparent post-operatively. As reported previously, intermediate monocytes were found to express significantly greater TLR4 and TLR5 than classical monocytes in healthy

controls. In addition, intermediate monocytes have also been shown to produce more IL-6 in response to LPS than other monocyte subpopulation (184). Therefore, it is possible that intermediate monocytes are the subpopulation responsible for increased IL-6 production in patients who develop post-operative SIRS due to the increase in TLR4 and TLR5 expression.

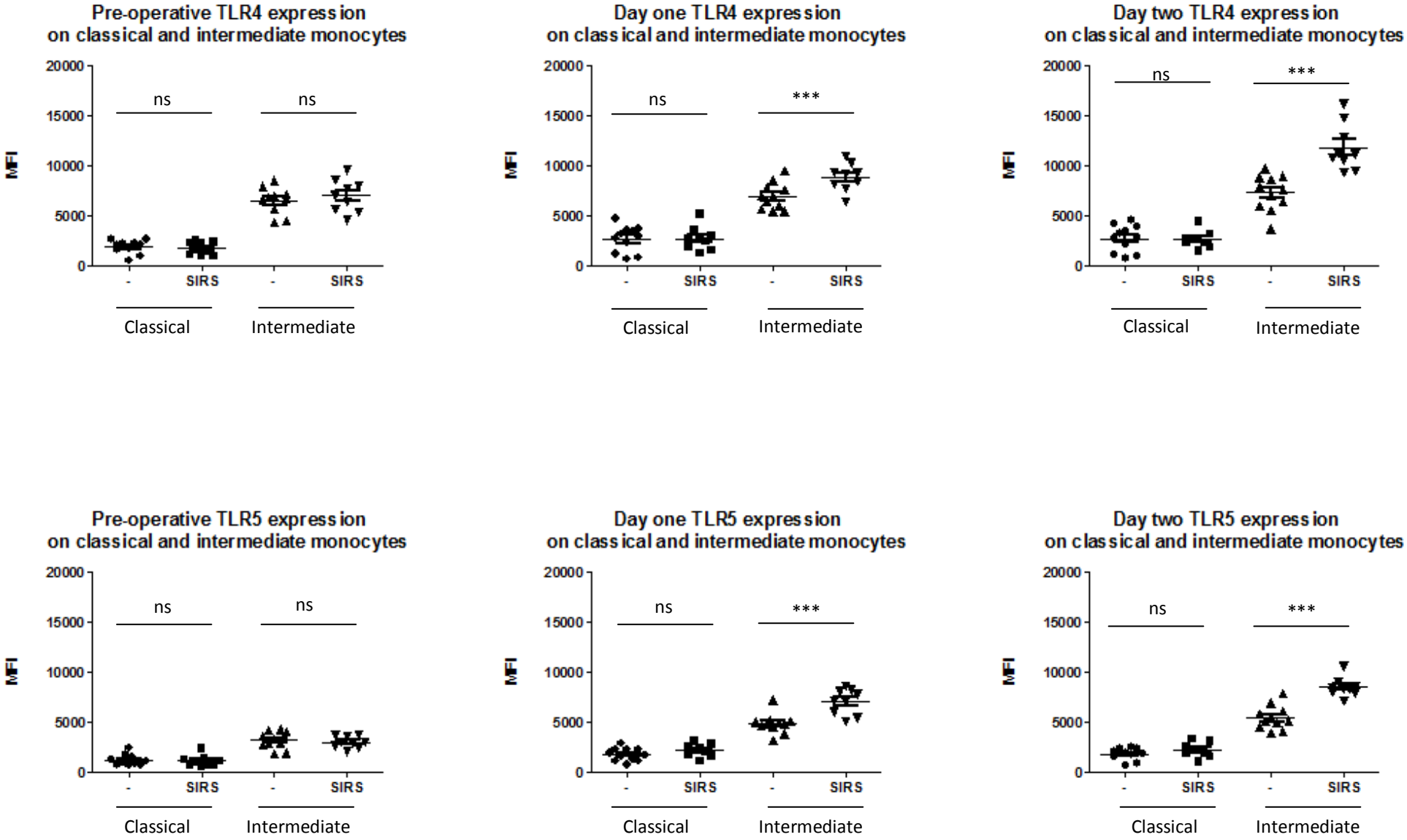


Figure 5.18. Pre-operative, day one and day two TLR4 and TLR5 expression on classical and intermediate monocytes

5.5 Discussion

5.5.1 Early post-operative monocyte TLR4 and TLR5 expression identified patients that go on to develop SIRS following major HPB surgery

The results from this series of experiments demonstrate that as early as post-operative day one, there is a significant increase in TLR4 and TLR5 expression on the surface of unstimulated monocytes in patients who go on to develop post-operative SIRS (Figure 5.2). The difference in receptor expression is even greater on post-operative day two in these patients. Conversely, in patients that have an uneventful post-operative recovery, there was no significant change in TLR4 and TLR5 expression when comparing pre-operative and early post-operative unstimulated monocytes (figure 5.2).

Patient serum did not alter TLR4 and TLR5 expression on healthy control monocytes. Further to this, stimulation of monocytes with LPS and flagellin did not lead to significant changes in TLR4 and TLR5 expression compared to unstimulated monocytes. Regarding the role of LPS in regulating TLR4 expression, the evidence is contradictory. A recent double-blinded, randomised crossover study demonstrated that low dose LPS did not increase TLR4 expression on monocytes in vivo (185). Another study demonstrated that LPS stimulation of monocytes led to increased TLR4 mRNA production but reduced receptor expression (186) (187). Another study found that treatment of corneal fibroblasts with LPS led to a dose dependent increase in TLR4 expression (188). Bacterial ligands can downregulate TLR5 mRNA production in bone-marrow derived dendritic cells (189) and both IFN-γ and GM-CSF downregulate TLR5 mRNA production in healthy human monocytes (190). The findings in this thesis did not identify any difference in TLR4 or TLR5 expression in response to LPS or flagellin. A possible reason for this is the dose of LPS and flagellin used, which was lower than that used in studies which demonstrated a change in TLR4 expression in response to

LPS. Another possible reason is that the kinetics of TLR4 and TLR5 expression in response to their agonists is unknown. I incubated PBMCs with LPS and flagellin for 24 hours, and any changes in monocyte TLR4 and TLR5 expression may be transient. Therefore, any potential feedback mechanisms to moderate TLR expression may have been activated and missed during this incubation period.

LPS- and flagellin-induced IL-6 production is correlated with TLR4 and TLR5 expression respectively (figures 5.4 and 5.5). Interestingly, TNF-α and IL-10 production were not increased in patients who develop SIRS in whom TLR4 and TLR5 expression were higher than in patients who did not. TLR expression and the production of some, but not all, inflammatory mediators are known to be associated. In a large series, human monocyte TLR4 expression was correlated to MIF production in response to LPS in immunosuppressed patients compared to healthy controls (191). However, this is not a consistent finding (192), although the dose of LPS in the latter study was 500 times greater than that used in this thesis and it is possible that the effect varies at different doses. Baseline messenger RNA transcript levels of TLR1, TLR2 and TLR4 were significantly greater in obese patients compared to lean controls. This was correlated with increased LPS induced TNF-α and IL-6 production (193). However, none of these studies looked at the association of these markers with clinical outcomes.

Although flow cytometry was chosen as experimental technique of choice, other techniques could have been used. The major benefit of flow cytometry was the ability to rapidly identify receptor (TLR) expression on a particular cell type (monocytes) that was part of a heterogenous population (PBMCs). Real-time quantitative polymerase chain reaction (qPCR) could have been used to evaluate potential differences in TLR4 and TLR5 mRNA levels between the groups. If time constraints had allowed it, quantification of TLR4 and TLR5 transcripts would have added useful data to this thesis, and this has subsequently been done by other members of the group (see attached draft manuscript). Western blotting is a commonly used technique that allows qualitative measurement of protein expression. Evaluation of monocyte cell surface TLR expression by western blotting has been

documented previously (194). Even band densitometry only provides semi-quantitative data in comparison to the median fluorescent intensity (MFI) generated by flow cytometry. In addition, monocytes need to be lysed for immunoprecipitated cell surface antibody staining. Lysis of monocytes may reduce the accuracy of TLR expression.

A strength of the protocol used in this thesis was that following agonist stimulation, supernatants were stored simultaneously for quantification of cytokine production by ELISA. Concurrently, PBMCs could be prepared to evaluate transcription factor phosphorylation (described in chapter 6).

Almost all the patients recruited to the study were diagnosed with an HPB malignancy (either primary tumour or liver metastases). Monocytes from healthy control volunteers were analysed and compared to pre-operative TLR expression from patients. As shown in this chapter, there was no significant difference in TLR expression between healthy controls and patients pre-operatively. A difficulty with this study design is that it is not possible to use healthy controls post-operatively. To compensate for this, patients who had an uneventful post-operative recovery were used for comparison against patients that developed SIRS. Another option would have involved using patients undergoing benign, uncomplicated surgery as post-operative controls (e.g. hernia repair, laparoscopic cholecystectomy). However, these patient have not faced the same surgical insult as those undergoing major HPB surgery, and the day case nature of uncomplicated surgery makes recruitment difficult.

Increased TLR4 and TLR5 expression on $CD14^{+}$ monocytes, suggests that the increased responsiveness of whole blood PBMCs to TLR agonists described in the previous chapter may be due to changes in monocyte signalling. We were able to study the expression of TLR4 and TLR5 on a subset of these patients for whom we had sufficient samples remaining. Changes in monocyte subset proportions and phenotype over the peri-operative period and their relationship to SIRS have not previously been studied.

Changes in the composition of monocyte subpopulations have been demonstrated in established sepsis (195). In patients with septicaemia, the $CD14^{dim}/CD16^{+}$ (intermediate) monocyte subpopulation was expanded compared to healthy controls. This was correlated with increased IL-6 levels in septic patients (195). Other work from the same group has demonstrated that intermediate monocytes produce a greater inflammatory response than $CD14^{+}/CD16^{-}$ (classical) monocytes following stimulation with LPS (196). In addition, bacterial ligand (LPS and Peptidoglycan) stimulation for 20 hours leads to expansion of the intermediate monocyte population (197). This is an expected finding as intermediate monocytes have been proposed to be the main producers of inflammatory cytokines within the monocyte population.

Changes in monocyte subpopulations in response to major surgery have not previously been reported. Prior to surgery, patients who went on to develop SIRS had a significantly greater proportion of intermediate monocytes than those who did not develop SIRS. Following surgery, the proportion of intermediate monocytes in both groups increased as an expected response to major surgery. In addition, TLR4 and TLR5 expression on intermediate monocytes was significantly greater than classical monocytes. This is in contrast to a previous study (197), although the study by Skinner *et al* evaluated TLR4 expression in septic patients rather than healthy patients undergoing a large surgical insult. It is possible that the complex crosstalk of immunological and inflammatory pathways in established sepsis leads to negative feedback and inhibition of TLR expression on intermediate monocytes.

5.5.2 Influences on changes in monocyte TLR4 and TLR5 expression in the peri-operative period

This chapter has demonstrated that patients who develop post-operative SIRS have changes in monocyte TLR4 and TLR5 expression early following major surgery. However, the reasons

for this change in TLR expression are not clear. It is possible that the trauma of the surgical insult 'primes' monocytes in susceptible patients' thereby increasing TLR4 and TLR5 expression. TLR4 expression on monocytes was increased in patients following major surgery, but this study did not investigate the role of changes in TLR4 expression with regards to identifying patients at high risk of developing SIRS (197). Another group found that monocyte TLR4 expression was not found to be significantly altered following major trauma compared to healthy controls (198). It was also shown that monocyte TLR2 expression increased immediately following cardio-pulmonary bypass and decreased on post-operative day seven in patients who have no complications (199). Although changes in TLR expression have been documented in the literature, no studies have evaluated the role of these changes as potential predictors of post-operative SIRS. In addition to this, the mechanism for the change in TLR expression has not yet been identified.

Anaesthetic agents may play a role alongside the surgical insult in the regulation of monocyte TLR expression. An ex-vivo study using umbilical vein endothelium showed that TLR2 and TLR4 expression was decreased following treatment with sevoflurane with a subsequent decrease in IL-6 and TNF-α production (200). This demonstrates the potential anti-inflammatory effects of sevoflurane and suggests that it may partially ameliorate the inflammatory response to major surgery. An active randomised trial in patients undergoing cardiac and vascular surgery will compare the incidence of SIRS following total intravenous anaesthesia to volatile anaesthesia. The results are currently awaited. Low dose per-operative intravenous methylprednisolone was found to reduce post-operative IL-6 production, length of intubation and ITU stay following cardio-pulmonary bypass in a randomised trial (201). A prospective randomised, double-blinded trial in post-operative patients with SIRS demonstrated that a single dose of dexamethasone following surgery did not inhibit the progression of SIRS. This indicates that the potential benefit of steroids may only occur if steroids are given in the peri-operative period, before SIRS has become established (202). As expected, it is likely that complex interactions between the surgical insult, anaesthetic agents and post-operative medications contribute to changes in monocyte TLR expression and cytokine production.

Protein synthesis is a complex process involving multiple essential steps that leads to protein production and function. It is possible that differences in transcription lead to the differences seen in TLR4 and TLR5 expression. RT-PCR could be used to assess differences in TLR4 and TLR5 mRNA levels and a previous study identified upregulation of TLR4 and TLR2 mRNA in patients with severe sepsis (203). Changes in TLR mRNA in the peri-operative period have not been evaluated previously and were not studied in this thesis. Differences in TLR4 translation in established sepsis have been studied. A single nucleotide polymorphism (rs11536889) affects TLR4 translation and was found to be more prevalent in ITU patients with established sepsis (204). Differences in TLR5 translation have not been evaluated. It is possible that post-transcriptional regulation of TLR4 and TLR5 could lead to the over expression seen in this thesis. There is evidence that LPS stimulation can lead to the rapid accumulation of Human antigen R (HuR) in the cytoplasm. Over expression of HuR has been shown to activate the translation of certain genes (205). Therefore, it is possible that patients who develop post-operative SIRS are more sensitive to LPS. As well as initiating the TLR4 signalling pathway, LPS in these patients may lead to increased accumulation of HuR, which may activate increased TLR4 translation and expression. It is not known whether HuR is involved in the regulation of TLR5. Another study demonstrated that stimulation of monocytes with Interferon-γ (IFNγ) up-regulated TLR4 expression, but downregulated TLR5 expression, thereby indicating that regulation of these two TLRs may be due to significantly different mechanisms (190). Mechanisms that cause TLR5 up-regulation on monocytes have not yet been elucidated. Although TLR5 was previously shown to be downregulated by IFN-γ, both TLR4 and TLR5 are known to be interferon stimulated genes (ISGs) (206). Therefore, increased activation of the JAK/STAT pathway by type I IFN binding may lead to differential TLR expression on the monocyte cell surface. It is possible that other innate immune sensors, including other TLRs, have increased cell surface expression in patients that develop post-operative SIRS. However, other TLRs have not been evaluated in this thesis.

5.5.3 Excluding inflammatory co-morbidities and changes in post-operative TLR4 and TLR5 expression

As described in 3.1.5.1, inflammatory co-morbidities have been shown to Increase TLR expression. Increased TLR2 and TLR4 expression has been identified in intestinal biopsy samples from inflammatory bowel disease patients (161). In addition, TLR2 expression on monocytes has been shown to be altered in patients with systemic lupus erythematosus (SLE) (162) and rheumatoid arthritis (163). Although TLR4 mRNA has been shown to be increased in hypertensive patients, this response is attenuated following anti-hypertensive treatment (164) and all patients with hypertension included in this study were on anti-hypertensive treatment. Hypertension was the most common co-morbidity in the study group and there was no difference in the incidence of hypertension between patients who had an uneventful recovery and those who develop SIRS. There are no reports in the literature of a correlation between TLR expression and hypercholesterolaemia.

5.5.4 Evidence of changes in post-operative TLR4 and TLR5 expression

TLR4 is the paradigm for toll-like receptor function and signalling. It has been studied extensively since its discovery in 1997 (112). However, the role of TLR4 in post-operative SIRS remains poorly understood. A number of small case series have been reported in the literature which have attempted to evaluate the expression and function of TLR4 following major surgery.

In 6 patients undergoing elective hip arthroplasty, there was no difference in TLR4 expression on peripheral blood monocytes in the first 12 days following surgery. However, this small cohort study did not compare patients who developed SIRS with those who did not (207). Interestingly, another study demonstrated that following elective arterial surgery,

TLR4 expression decreases immediately following surgery (208). The results presented in this chapter do not support this initial finding. However, Versteeg et al found that TLR2 and TLR4 expression was increased 24 hours following surgery in keeping with the TLR4 findings in this chapter. In addition, this study did not investigate the difference in TLR4 expression between patients with complications and those without (208). Another group investigated changes in monocyte subpopulations and TLR4 expression following hepatectomy. They found that major surgery increased TLR4 expression on non-classical ($CD14^{+}/CD16^{+}$) monocytes (209). Although we did not see this effect in our study and Efron *et al* did not compare patients who developed SIRS with those who had no complications. The results presented in this thesis show that TLR4 expression in patients with an uneventful recovery was unchanged in the post-operative period. However, when combining all patients together (as done in the studies above), there was a significant increase in monocyte TLR4 expression in all patients on post-operative days one and two. Another case series investigated the role of changing TLR4 expression on monocytes in patients undergoing orthotopic liver transplantation (OLT). Specifically, this study evaluated the relationship between TLR4 expression and post-operative acute lung injury (ALI). The same dataset was also used to assess the relationship between early post-operative TLR4 expression and SIRS. They demonstrated that TLR4 expression is significantly increased on the first post-operative day in OLT patients that go on to develop ALI and SIRS (15) (210). The methods used in these studies were different to those used in this thesis. Both studies stained whole blood with TLR2, TLR4 and CD14 antibodies and then added red cell lysis buffer prior to analysis. In addition, only 4ml of blood was collected at each time point. 20ml of blood was collected at each time point in this thesis to maximise monocyte numbers. This allowed a larger number of events to be recorded, thereby increasing the accuracy of flow cytometry analysis. The findings presented in this chapter are in agreement with the results from these papers, indicating that increased TLR4 expression in the early post-operative period could identify patients at high risk of developing post-operative SIRS. However, these studies did not identify differences in TLR5 expression or the TLR signal transduction pathway.

A study investigating changes in TLR4 function in patients undergoing gastrointestinal surgery demonstrated that high pre-operative expression of TLR4 reduced the capacity of

monocytes to produce inflammatory cytokines in response to LPS. They also stated that 4 patients with high pre-operative TLR4 expression developed post-operative infections (21). The results presented in this chapter did not reach the same conclusions as pre-operatively, there was a positive correlation between TLR4 expression and IL-6 production. A possible explanation for the difference in these results is the dose of LPS used. Ono et al used a concentration of LPS 500 times greater than that used in this thesis. The high dose of LPS may saturate monocyte TLR4 receptors and paradoxically impair LPS-induced signal transduction and subsequent cytokine production. The lower dose of LPS used in this thesis may have allowed normal signal transduction to continue after LPS binding.

Ileal mucosa from patients with Ulcerative Colitis (UC) who had undergone ileal pouch reconstruction was compared with control mucosa from colorectal cancer patients. TLR4 mRNA was significantly up-regulated in UC patients both with and without active pouchitis, indicating that post-operative changes in TLR4 expression may be involved in local inflammatory processes (118). A small case series identified different trends in TLR4 expression in patients with and without rejection following renal transplantation. This study identified that TLR4 expression increases in the early post-operative period of patients that develop organ rejection, indicating that innate immune sensing may play a role in this complex immunological process (211). These findings of deleterious inflammatory effects corresponding with elevated TLR4 expression following surgery are in keeping with the findings presented in this thesis.

The finding in this thesis that monocyte TLR4 expression is significantly increased in patients that go on to develop post-operative SIRS in the early post-operative period is corroborated by similar results from number of studies published in the scientific literature. However, this study has a larger cohort of patients than any published previously. This thesis has also attempted to identify a mechanism by which increased TLR4 and TLR5 expression leads to exaggerated cytokine production.

TLR5 binds flagellin, and is therefore the only known toll-like receptor to bind both gram-positive and gram-negative bacteria. The study by Toiyama *et al* highlighted earlier demonstrated that normal ileal mucosa expressed high levels of TLR5 mRNA. This was an expected finding as TLR5 is known to be highly expressed in intestinal epithelium. Interestingly, in both active pouchitis and in patients that have had ileal pouch reconstruction, TLR5 mRNA levels are significantly reduced (118).

To my knowledge, there are no published studies in the scientific literature that have evaluated the role of monocyte TLR5 expression as a potential biomarker for post-operative SIRS. The data presented in this thesis demonstrated that monocyte TLR5 expression was significantly greater in patients who go on to develop post-operative SIRS in the early post-operative period. As shown earlier, monocytes from patients who develop SIRS have significantly greater expression of both TLR4 and TLR5 and it may be the case that the expression of other TLRs is altered immediately after surgery in patients who develop SIRS. This suggests that following the surgical insult of a major operation, innate immune cells (such as monocytes) are 'primed' for an exaggerated response to bacterial ligands due to over expression of various TLRs which may contribute to the development of post-operative SIRS.

Therefore, TLR4 and TLR5 expression on peripheral monocytes on post-operative day one and day two can be used to identify patients that are at high risk of developing post-operative SIRS.

6. Quantification of transcription factor phosphorylation in monocytes

6.1 Introduction

The data presented thus far suggest that innate immune dysfunction in monocytes may be able predict SIRS in patients undergoing major HPB surgery. Upregulation of TLR4 and TLR5 is associated with increased production of IL-6 in response to LPS and flagellin. In order to evaluate intracellular signalling mediators of these pathways, I have modified the FACS-based Phosflow technique to enable analysis of multiple transcription factors in different cell types within a heterogenous population. Phosflow is a technique that utilises intracellular flow cytometry to evaluate protein phosphorylation. Cell populations of interest can be isolated by surface antibody staining and following permeabilisation, changes in protein activation assessed. To reduce intra-experimental variability and conserve patient samples, I assessed three transcription factors (NF-κB, ERK1/2 and STAT1) known to be integral to inflammatory signalling, simultaneously. Following 15 minutes' stimulation with LPS, flagellin and IFN2α, the PBMCs were fixed to prevent further changes in protein phosphorylation. As shown in this chapter, stimulation with LPS, PMA and IFN2α for 15 minutes produced a significant change in NF-κB, ERK1/2 and IFN2α phosphorylation compared to unstimulated baseline phosphorylation. The major benefit of using multiplex Phosflow is the ability to assess concurrent changes in multiple transcription factors in an identical population of cells.

NF-κB is a ubiquitous protein complex that plays a key role in inflammation (124). ERK1/2 (or classical MAP kinases) is involved in inflammatory cytokine production and cell division among other cellular processes and represents a key NF-κB-independent transcription factor. The response to TLR agonists is amplified in part by the production of type I interferons which principally signal through STAT proteins (115).

The data presented in this chapter identified a significant increase in LPS and flagellin-stimulated NF-κB phosphorylation in monocytes on post-operative days one and two in patients who develop SIRS compared to patients with an uneventful recovery, in keeping with the TLR expression and cytokine results. Concomitantly, there was a significant reduction in ERK1/2 phosphorylation on post-operative day two. In addition, I have identified a pre-operative difference between the two groups of patients in that interferon-2α stimulated STAT1 phosphorylation was significantly greater in patients who developed SIRS, although there was no significant difference between the groups in STAT1 phosphorylation post-operatively.

6.1.1 Identification of the monocyte (CD14+) population using flow cytometry for quantification of transcription factor phosphorylation

Monocytes were identified by $CD14^+$ positive antibody staining (Figure 6.1). Baseline transcription factor phosphorylation was compared to the experimental conditions (unstimulated, LPS-, flagellin- and IFN2α-stimulation) and results are expressed as the percentage change in phosphorylation from baseline (Figure 6.2). To reduce intra-experimental variability, NF-κB, ERK1/2 and STAT1 phosphorylation was analysed within the same FACS tube for each experimental condition.

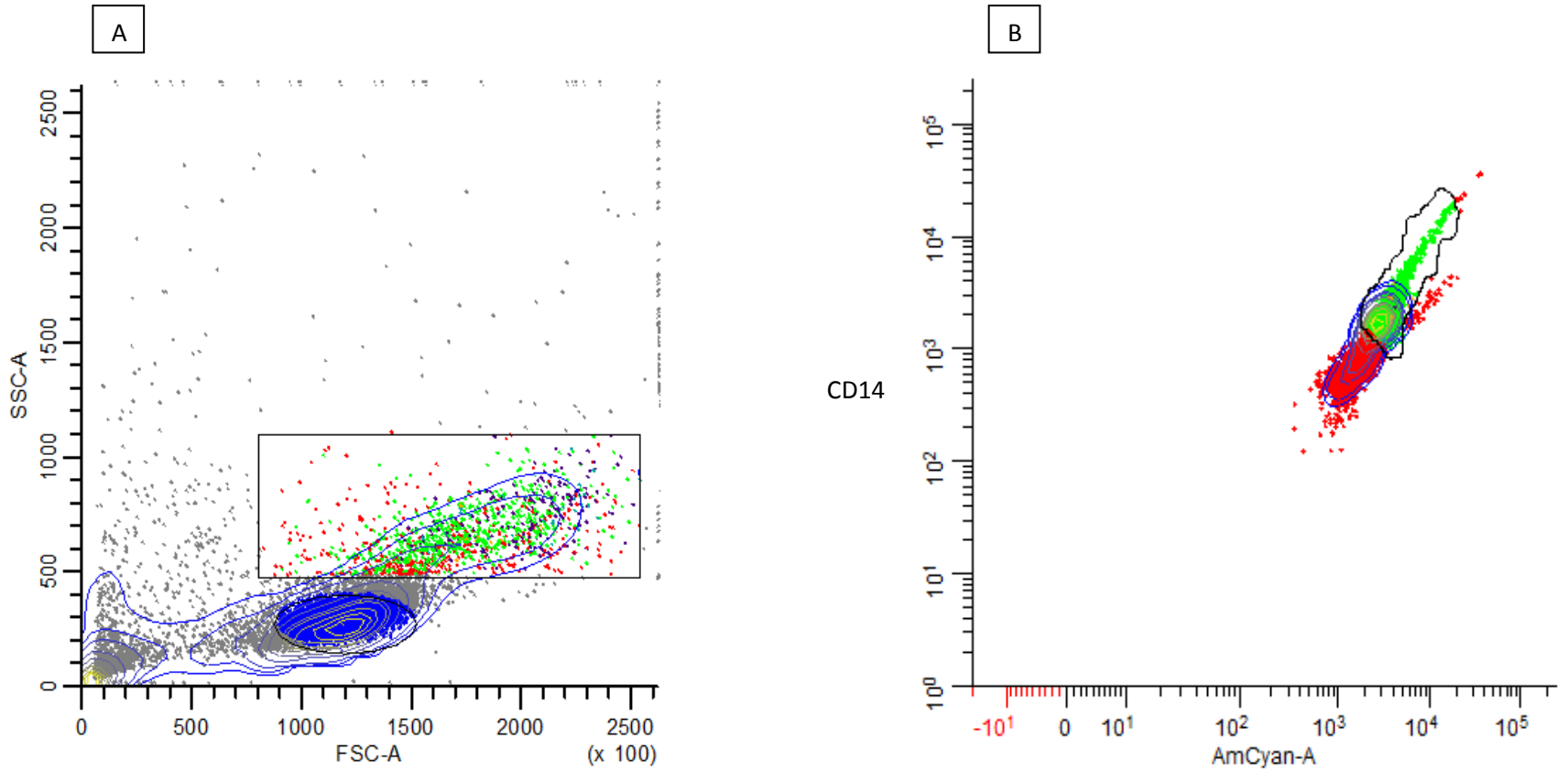


Figure 6.1. CD14$^+$ cells (monocytes) were identified on morphological (A) and CD14 antibody staining (B)

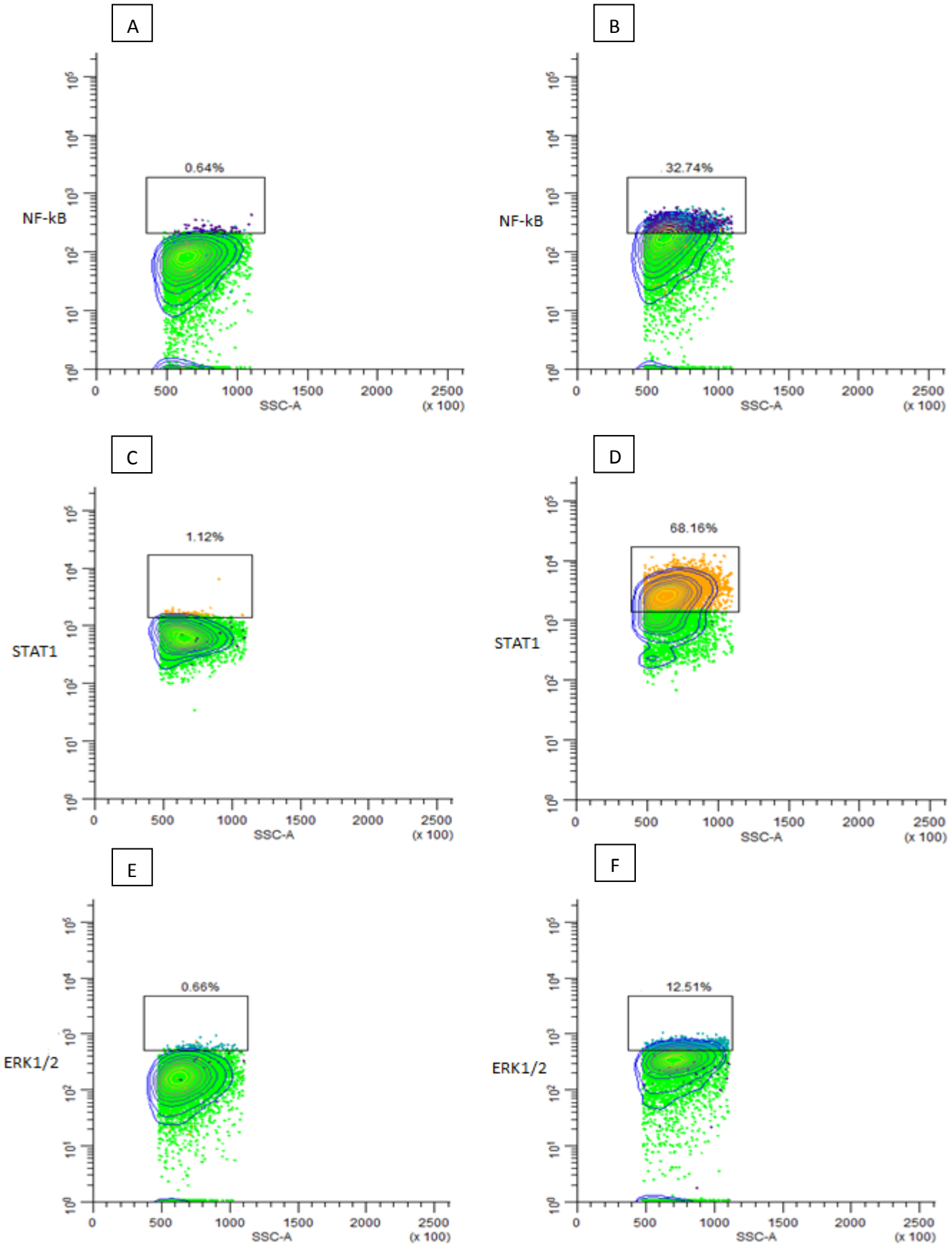


Figure 6.2. Isotype controls for NF-κB (A), STAT1 (C) and ERK1/2 (E) on $CD14^+$ cells show background phosphorylation. The percentage change in NF-κB (B), STAT1 (D) and ERK1/2 (F) phosphorylation on $CD14^+$ cells was measured following LPS (B), IFN2α (D) and PMA (F) stimulation respectively.

6.2 Quantification of NF-κB and ERK1/2 phosphorylation on $CD14^+$ cells

In unstimulated monocytes, NF-κB and ERK1/2 phosphorylation were minimal in all patients. In particular, there was no significant difference in NF-κB phosphorylation between patients who develop SIRS and those who have an uneventful recovery pre-operatively, on day one and day two post-operatively. Longitudinally, there were no significant changes in unstimulated monocyte NF-κB or ERK1/2 phosphorylation in the peri-operative period (Figure 6.5 and 6.6).

Following fifteen minutes' stimulation with LPS, there was no significant difference in pre-operative NF-κB phosphorylation between patients who develop SIRS and those who have an uneventful recovery. However, and in keeping with the TLR expression data on post-operative day one, LPS-stimulated monocytes from patients who develop SIRS had significantly greater NF-κB phosphorylation (16.34% change from baseline; $p=0.005$) compared to patients who have an uneventful recovery (3.76% change from baseline). There was a further significant increase (25.6% change from baseline; $p<0.001$) in LPS-stimulated monocyte NF-κB phosphorylation on day two in patients who develop SIRS compared to patients who have an uneventful recovery (2.18% change in baseline) (Figure 6.5).

A similar pattern was seen in flagellin-stimulated monocytes, with no pre-operative differences in NF-κB phosphorylation and increased NF-κB phosphorylation in flagellin stimulated monocytes on day one (No SIRS change in baseline 4.4% vs SIRS change in baseline 15.1%; $p<0.005$) and day two (No SIRS change in baseline 2.8% vs SIRS change in baseline 22.3%; $p<0.002$) in patients who developed SIRS compared to those who had an uneventful recovery (Figure 6.5).

In contrast to the increase seen in NF-κB phosphorylation following TLR agonist stimulation, there was a significant decrease in unstimulated ERK1/2 phosphorylation on post-operative day two in patients who developed SIRS compared to those who have an uneventful recovery. This was seen in unstimulated (No SIRS change in baseline 5.9% vs SIRS change in baseline 1.3%; $p<0.04$), LPS- (No SIRS change in baseline 5.9% vs SIRS change in baseline 2.2%; $p<0.03$) and flagellin (No SIRS change in baseline 5.9% vs SIRS change in baseline 1.7%; $p<0.03$) stimulated monocytes (Figure 6.6).

In summary, following LPS and flagellin stimulation of monocytes from patients who develop SIRS, there is a simultaneous increase in NF-κB phosphorylation and decrease in ERK1/2 phosphorylation on post-operative day two.

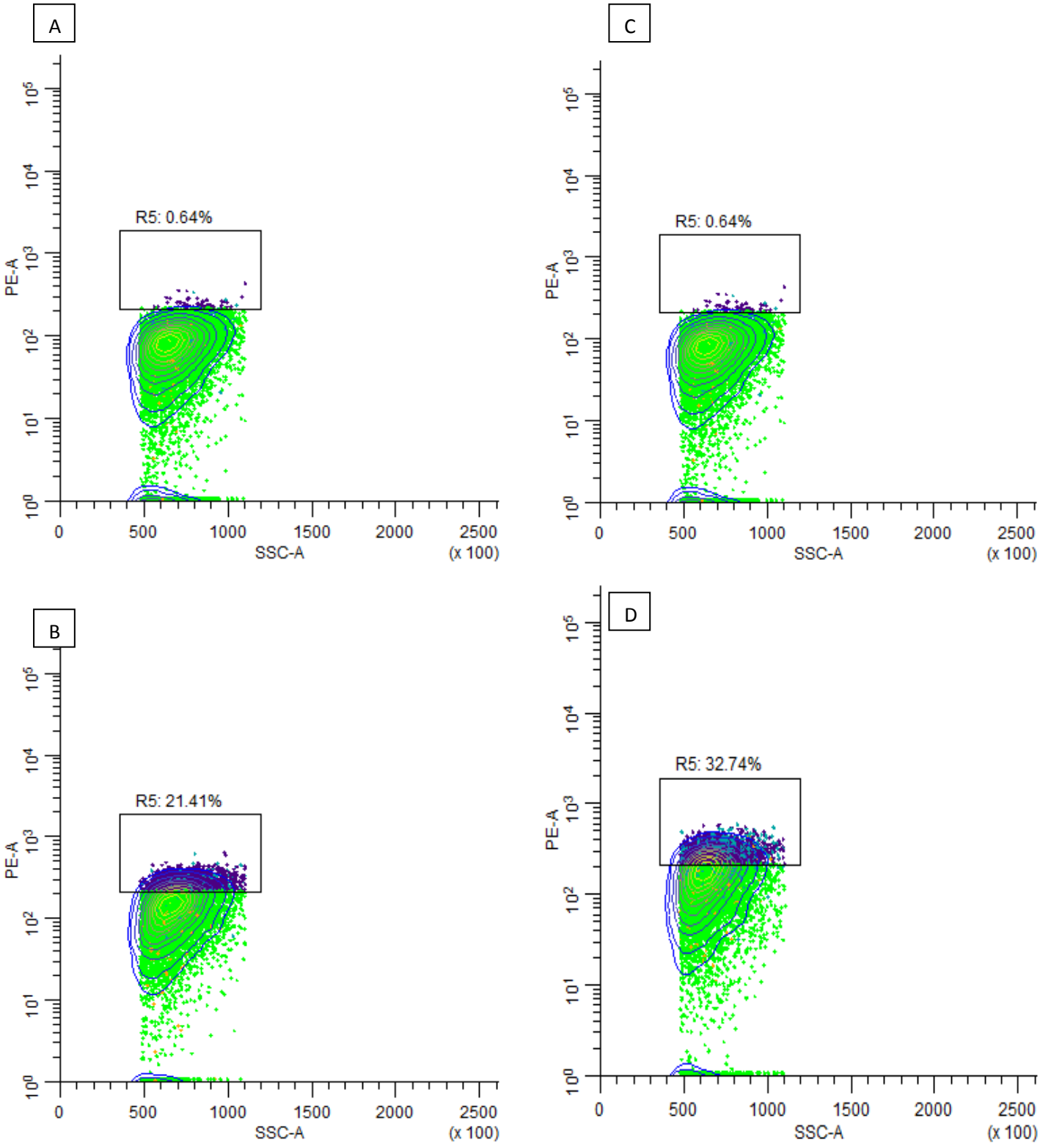


Figure 6.3. FACS plots demonstrating the increase in NF-κB phosphorylation in monocytes in response to LPS (B) and flagellin (D) in a patient who developed SIRS on day six following PPPD.

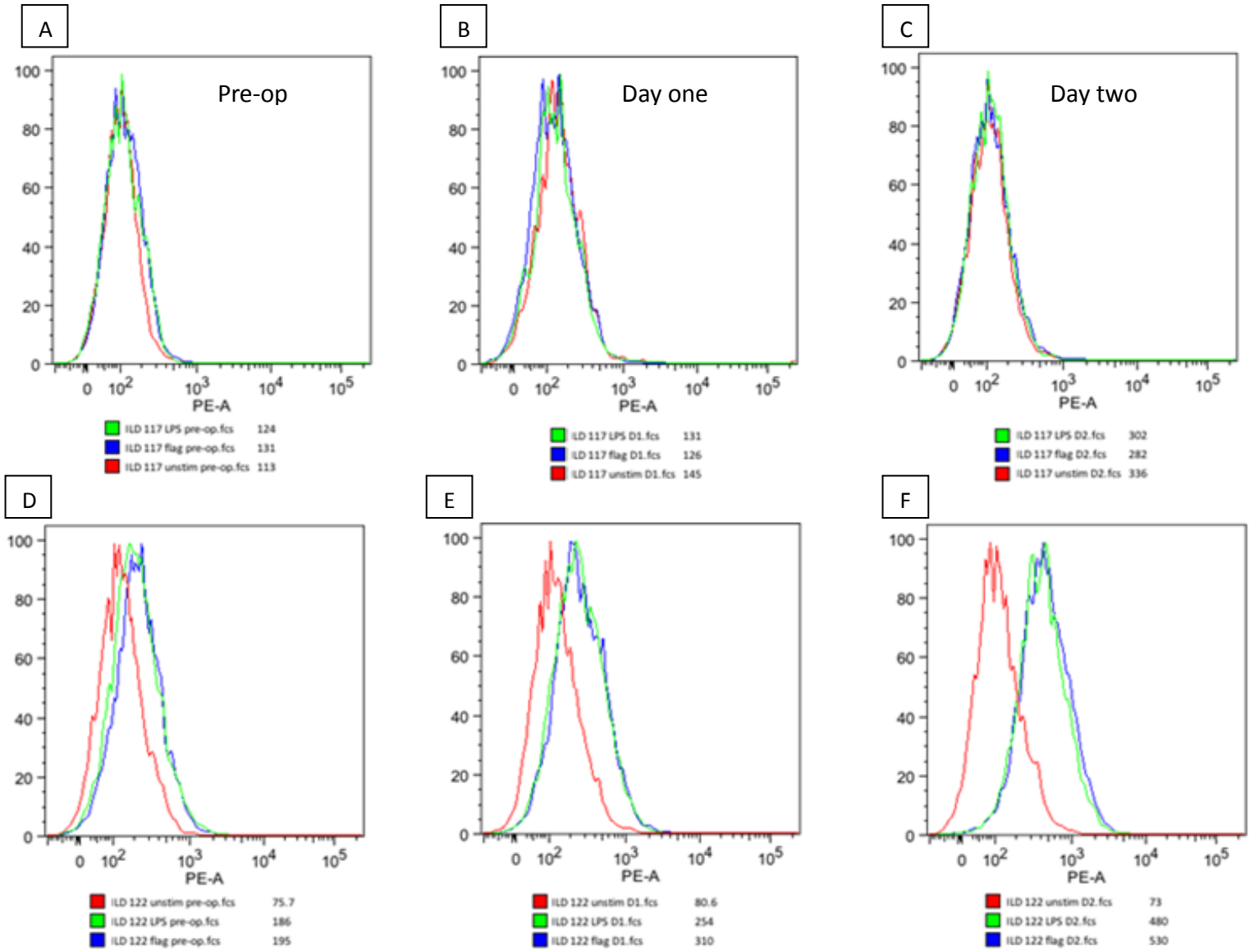


Figure 6.4. Phosflow histograms demonstrating the change in unstimulated (red), LPS (blue) and flagellin (green) stimulated monocyte NF-κB phosphorylation in a patient without complications (A-C) and a patient who developed SIRS on day four (D-F) following PPPD

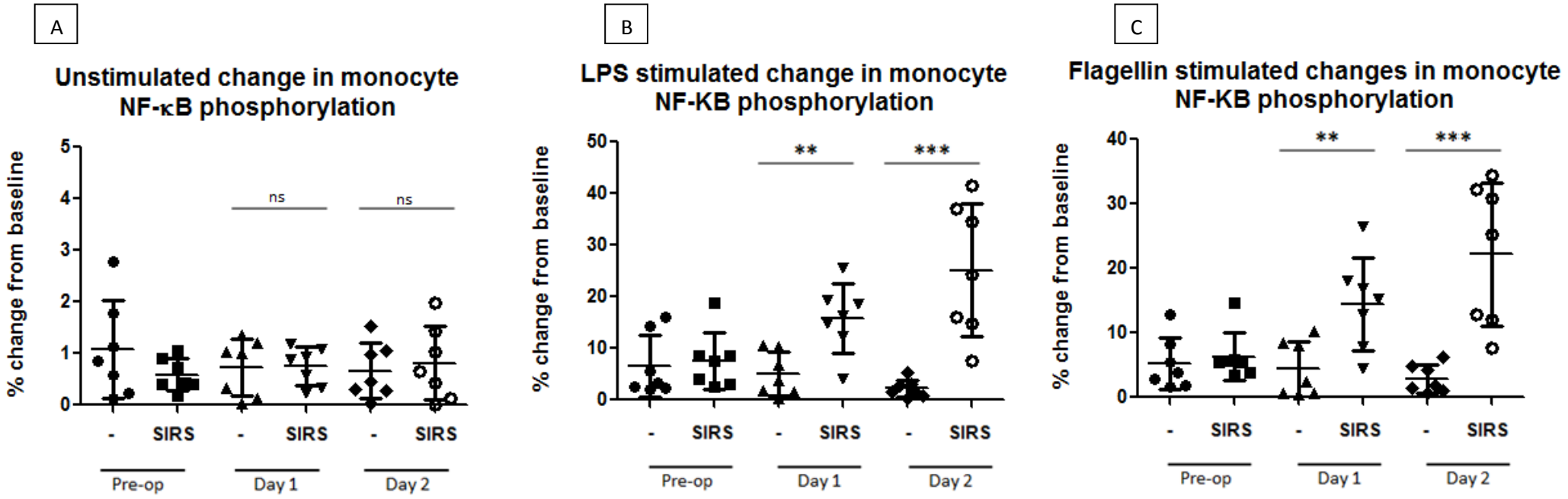


Figure 6.5. Unstimulated (A), LPS-stimulated (B) and flagellin-stimulated (C) NF-κB phosphorylation before surgery (pre-op) and on day one (Day 1) and day two (Day 2) following surgery.

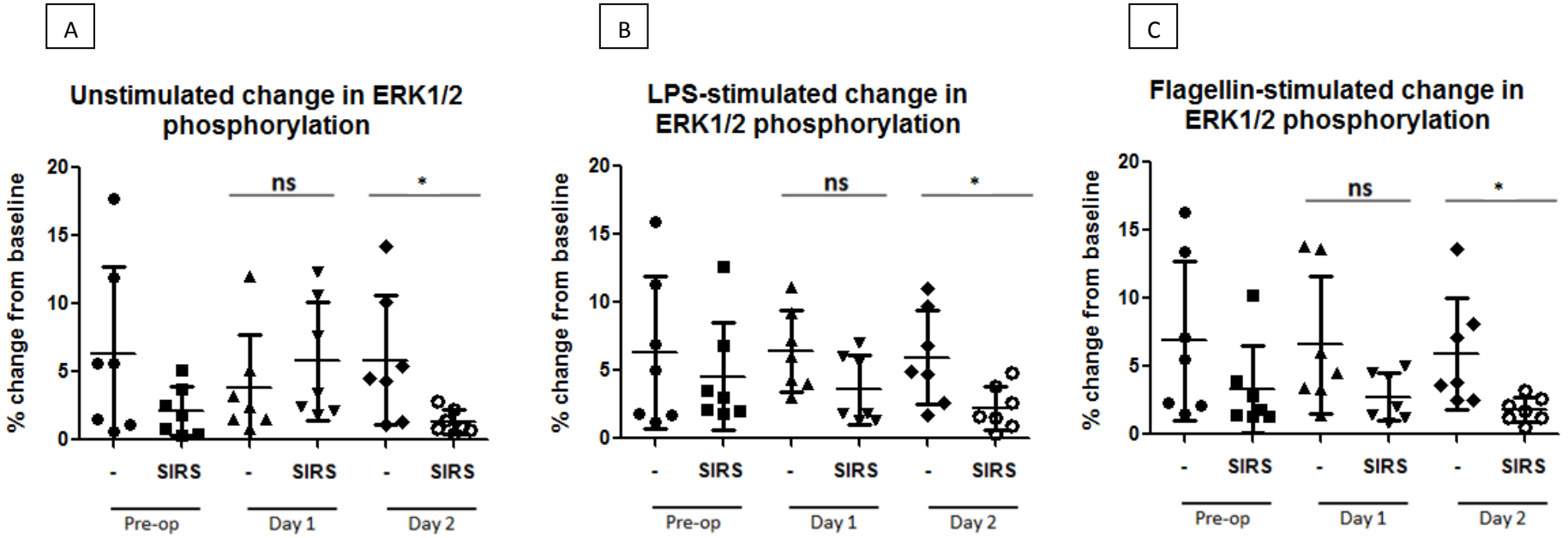


Figure 6.6. Changes in unstimulated (A), LPS (B), flagellin (C) stimulated monocyte ERK1/2 phosphorylation before surgery (Pre-op) and on day one (Day 1) and day two (Day 2) following surgery in patients who have an uneventful recovery (-) and those who develop SIRS (SIRS).

6.2.1 Correlation between TLR4 and TLR5 expression and NF-κB phosphorylation

The finding that TLR expression, NF-κB phosphorylation and IL-6 production are all increased in patients who develop SIRS suggests that these processes may be linked. To determine if these events were associated in patients who went on to develop post-operative SIRS, TLR4 and TLR5 expression were plotted against LPS- and flagellin stimulated NF-κB phosphorylation respectively. A positive correlation was found between TLR expression and NF-κB phosphorylation (Figure 6.7). This was statistically significant pre-operatively and on both post-operative days for both TLRs and their respective agonists. This is consistent with the finding that monocyte TLR4 and TLR5 expression was associated with IL-6 production.

6.3 Quantification of STAT1 phosphorylation on monocytes

In contrast to the post-operative changes seen in NF-κB and ERK1/2 phosphorylation in patients who develop SIRS, there were no differences seen in unstimulated monocyte STAT1 phosphorylation following surgery between the groups of patients. This was also the case following LPS-, flagellin- stimulation (figure 6.9).

Interestingly, patients who developed SIRS had greater IFN2α stimulated monocyte STAT1 phosphorylation pre-operatively (43.9% change from baseline) compared to those who had an uneventful recovery (25.5% change from baseline; p=0.02) (figure 6.9). This difference was not present on post-operative days one and two. In both groups, IFN2α stimulated monocytes produced significantly greater STAT1 phosphorylation than unstimulated, LPS and flagellin stimulated monocytes.

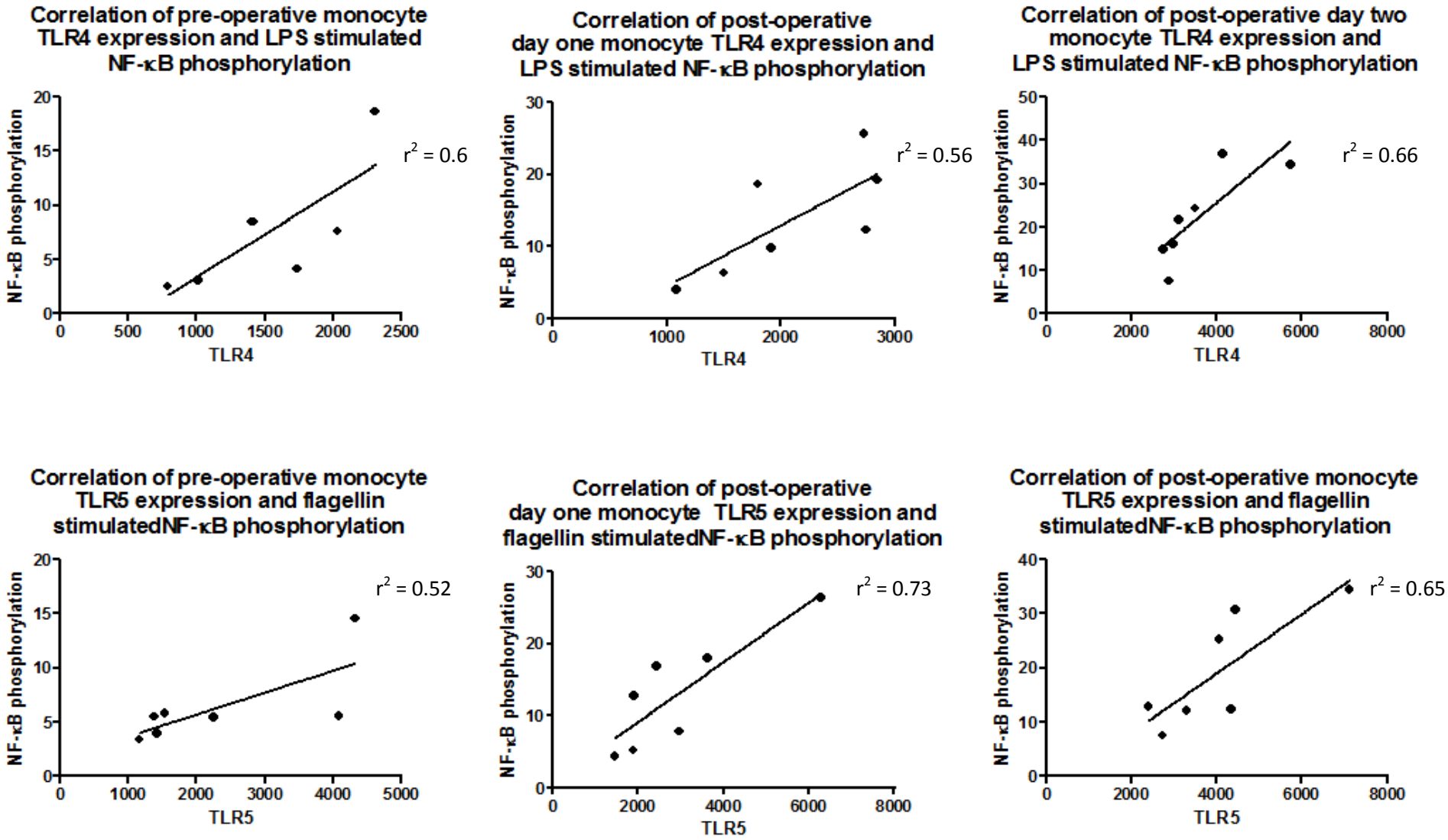


Figure 6.7. The correlation between the pre-operative, post-operative day one and day two TLR4 expression and LPS-stimulated NF-κB phosphorylation and TLR5 expression and flagellin stimulated NF-κB phosphorylation in patients who develop SIRS. The r^2 coefficient is presented with each graph ($p<0.005$ for all correlations).

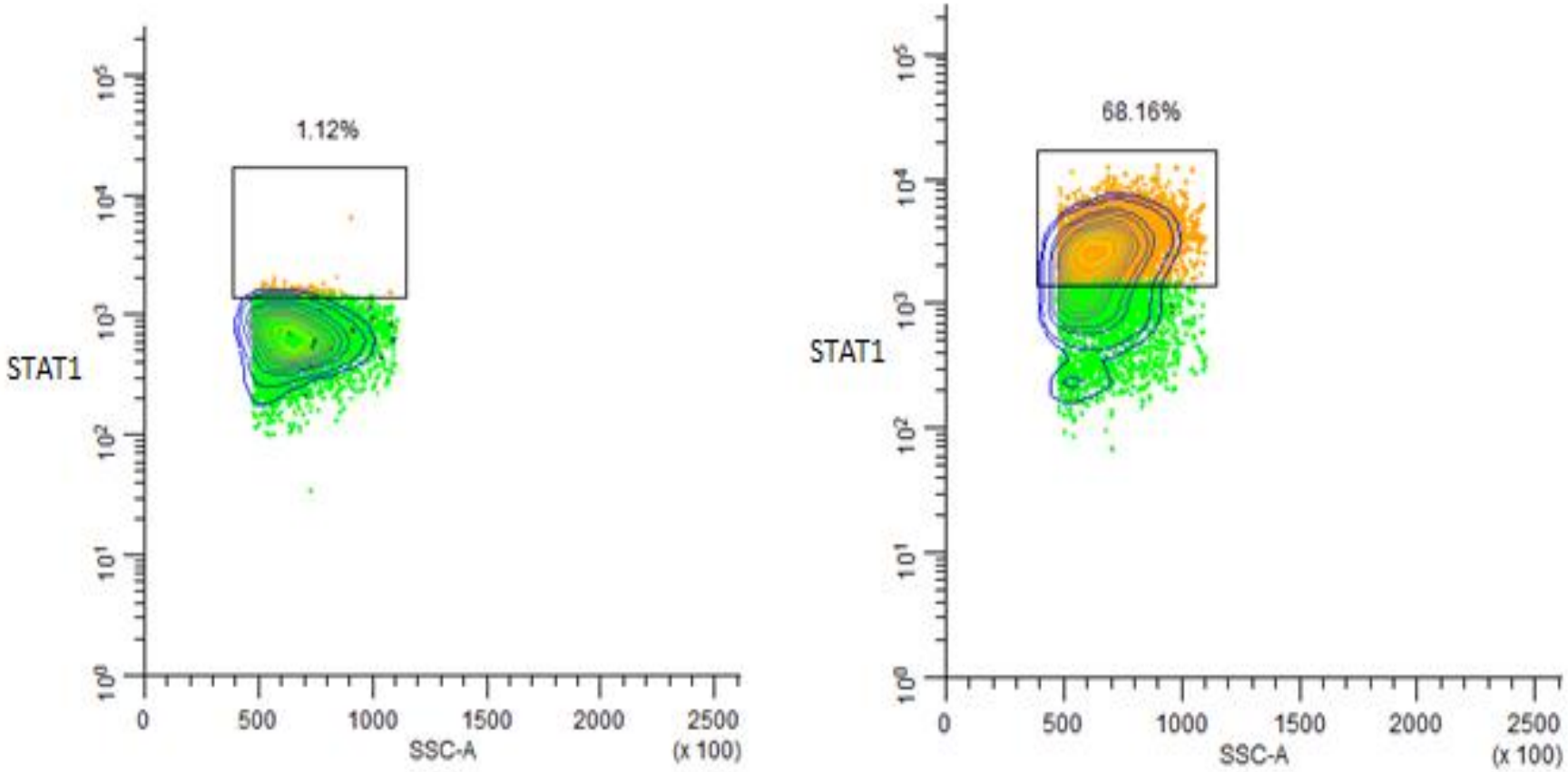


Figure 6.8. FACS plot demonstrating the change in STAT1 phosphorylation between isotype control (A) and IFN2α stimulated monocytes (B) in a pre-operative sample from a patient who went on to develop SIRS following surgery.

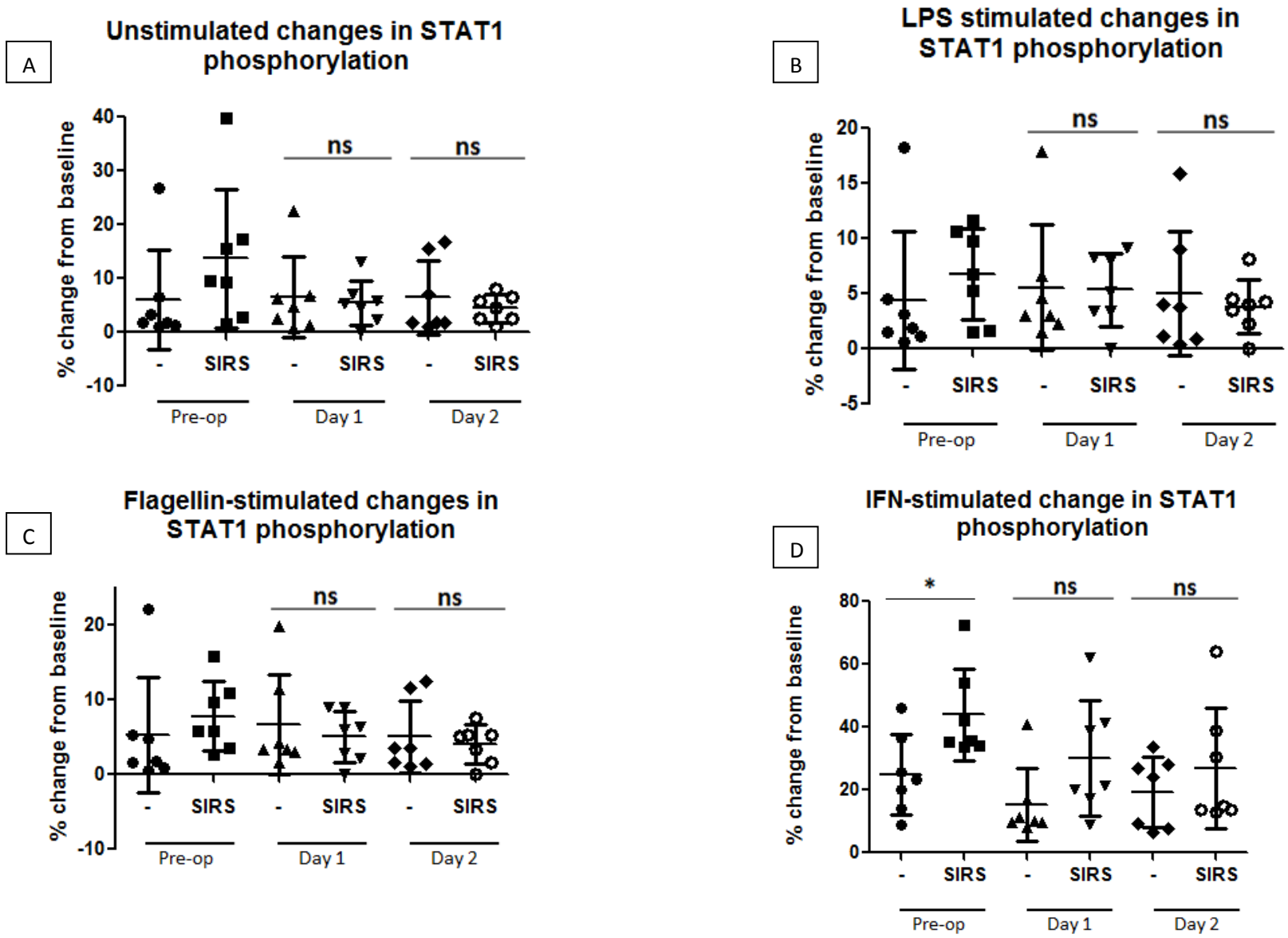


Figure 6.9. Changes in unstimulated (A), LPS (B), flagellin (C) and IFN (D) stimulated monocyte STAT1 phosphorylation before surgery (Pre-op) and on day one (Day 1) and day two (Day 2) following surgery.

6.4 Discussion

6.4.1 Multiplex phosflow – a new way to measure transcription factor phosphorylation

To my knowledge, this is the first time multiplex phosflow has been used to evaluate changes in numerous transcription factors simultaneously involved in the inflammatory signalling pathway in response to major surgery. Given the importance of inflammatory dysfunction in many disease processes (e.g. Rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease), this assay could be used to assess changes in key transcription factors in numerous conditions. This technique is expensive due to the cost of antibodies and permeabilisation buffer. The NF-κB antibody was specific to the p65 (REL-A) protein. The p65 subunit contains the transactivation domain, which is responsible for the interaction with the inhibitor IκB. In monocytes, the p50/p65 heterodimer is located within the cytoplasm in a complex with IκB. This complex prevents nuclear translocation and activity of NF-κB. In response to stimuli such as LPS, IκB is phosphorylated at critical residues and induces dissociation of the IκB/NF-κB complex, allowing the free heterodimeric NF-κB to translocate to the nucleus and initiate inflammatory cytokine production.

6.4.2 Changes in transcription factor phosphorylation identified patients that go on to develop SIRS following major HPB surgery

The results presented in this chapter demonstrate that LPS and flagellin stimulated monocytes from patients who develop post-operative SIRS produce increased NF-κB phosphorylation compared to patients who develop no complications. Conversely, on post-operative day two, monocytes from patients who develop SIRS produce less ERK1/2 phosphorylation than monocytes from patients who have an uneventful recovery. Pre-

operatively, IFN2α stimulated monocytes from patients who develop post-operative SIRS produced significantly more STAT1 phosphorylation than patients who had an uneventful recovery.

NF-κB is a key modulator of inflammation and is found in almost all cells. Activation of TLR signalling pathways leads to NF-κB phosphorylation, translocation to the nucleus and subsequent inflammatory cytokine production. Therefore, given that I have identified increased TLR4 and TLR5 expression and increased cytokine production in patients who develop SIRS, the finding of increased NF-κB phosphorylation is in keeping with these earlier results. The association of these events is supported by the positive correlations between TLR expression and NF-κB phosphorylation. Interestingly, there was a concurrent decrease in ERK1/2 phosphorylation in patients who develop SIRS. This suggested that there may be a skew in transcription factor phosphorylation and that the balance between NF-κB and ERK1/2 is disrupted in favour of NF-κB. It would be important to assess activation status of other transcription factors known to mediate TLR signalling and to examine the gene expression signatures from these monocytes.

The importance of changes in NF-κB activity in SIRS has been studied previously. A human series (213) identified significantly greater intra-nuclear NF-κB expression by flow cytometry in trauma patients with established SIRS compared to healthy controls. The data presented in this chapter support this finding. A murine macrophage cell line (J774a.1) treated with glucan phosphate (which inhibits LPS binding) produced significantly less NF-κB activation (214) than untreated cells. In a mouse model, the use of decoy oligodeoxynucleotides (ODNs), which inhibit NF-κB phosphorylation, reduced pulmonary inflammation, pulmonary vascular permeability and pro-inflammatory cytokine production in mice after caecal ligation and puncture (CLP). The authors acknowledged the possibility of NF-κB inhibitors reducing the severity of SIRS (215). An animal model evaluating the role of selective NF-κB inhibitors following colonic anastomosis found that treatment with parthenolide or resveratrol (selective NF-κB inhibitors) led to increased fibroblast activity and collagen deposition at the site of anastomosis (214). These studies indicate the importance of NF-κB

inhibitors to potentially attenuate SIRS following surgery. The ability of selective NF-κB inhibitors to improve outcomes in patients with established SIRS and sepsis have not yet been evaluated in clinical trials. Taken with the results presented in Chapter 5, in patients who develop SIRS, changes in monocyte TLR4 and TLR5 expression on the first day following major surgery appear to drive increased NF-κB phosphorylation following LPS and flagellin stimulation. This suggests that inhibition of NF-κB phosphorylation remains an attractive therapeutic target to reduce cytokine production and subsequently the post-operative incidence of SIRS.

The role of ERK1/2 in post-operative SIRS has not been fully characterised. In a CLP-induced acute lung injury mouse model, increased levels of ERK1/2 were shown on western blot (217) as well as increased IL-6 production and increased microvascular permeability. Treatment with Penehyclidine hydrochloride (an anticholinergic drug) was shown to attenuate ERK1/2 expression and reduce IL-6 production. Another animal study presented different results. It identified reduced ERK1/2 phosphorylation in rats with a chronic intra-abdominal abscess (218). IL-6 production was not measured in this study. This may indicate differences in ERK1/2 expression between acute and chronic inflammation. The results presented in this chapter are in keeping with this finding; although a key difference is that the animal model used by Vary et al had established sepsis, whereas reduced ERK1/2 phosphorylation was seen in patients who went on to develop SIRS. As NF-κB phosphorylation is significantly increased in patients who went on to develop SIRS, it is possible that NF-κB is preferentially phosphorylated, with a subsequent reduction in ERK1/2 phosphorylation. This inverse correlation of NF-κB and ERK1/2 phosphorylation has not been previously documented in post-operative SIRS.

STAT1 phosphorylation is activated following interferon binding and subsequently leads to the production of interferon stimulating genes (ISGs), which include TLR4 and TLR5 (206). Another study in mice demonstrated that $STAT1^{-/-}$ mice are more resistant to LPS-induced shock (219). This is most likely due to disruption of the IFNAR signalling pathway. Another study corroborated these findings, as they demonstrated that survival rates in $STAT1^{-/-}$ mice

were significantly greater than in wild-type in response to caecal ligation and puncture (220). They also showed that IL-6 production was significantly reduced in STAT1$^{-/-}$ mice, indicating that STAT1 activation is involved in inflammatory cytokine production and may play an important role in systemic inflammation following surgical insult. The role of STAT1 in post-operative SIRS has not been evaluated. Yohimbine (α_2-adrenergic receptor antagonist) and Berberine (a natural isoquinoline alkaloid) pre-treatment was shown to improve survival in septic mice in association with reduced NF-κB activation and ERK1/2 and STAT1 phosphorylation (221). However, STAT1 phosphorylation was evaluated only one hour after LPS challenge, which may only capture the early activation of STAT1 signalling, as was shown in the results presented here. Evaluation of changes in STAT1 phosphorylation in the peri-operative period has not previously been reported. The results presented in this chapter identified that IFN2α stimulated pre-operative STAT1 phosphorylation was significantly increased in patients. There was no significant difference in IFN2α stimulated STAT1 phosphorylation post-operatively. However, pre-operative levels of STAT1 phosphorylation were higher than following surgery in both groups of patients suggesting that major surgery reduced STAT1 phosphorylation in all patients.

Given that animal studies have shown the resistance of mice to endotoxic shock in the absence of STAT1, patients with elevated STAT1 phosphorylation pre-operatively may be at higher risk of developing post-operative SIRS and sepsis and is supported by the results in this chapter. The mechanism of upregulation of STAT1 phosphorylation in these patients remains to be elucidated. Assessment of other transcription factors involved in inflammation (e.g. AP-1, IRF3) would provide further understanding of the role of TLR signalling in identifying patients at high risk of developing post-operative SIRS.

Together with the results in Chapters 4 and 5, these results may indicate a pathway that can be used to identify patients at high risk of developing post-operative SIRS following HPB surgery. In patients who develop SIRS, circulating monocytes may be hyper-responsive to STAT1. Increased STAT1 phosphorylation leads to the transcription and production of monocyte TLR4 and TLR5 which in turn increases in expression in response to major surgery.

Increased TLR expression allows greater TLR agonist binding which leads in turn to greater NF-κB phosphorylation and a concurrent decrease in ERK1/2 phosphorylation. This suggests preferential activation of NF-κB and subsequent pro-inflammatory cytokine production (specifically IL-6). These changes occur within 36 hours of major surgery. In addition to the findings in TLR signalling, patients who develop SIRS have a greater proportion of $CD14^{+}/CD16^{+}$ (intermediate) monocytes pre-operatively. These monocytes were found to have significantly greater TLR4 and TLR5 expression than $CD14^{+}/CD16^{-}$ (classical) monocytes. This indicates that although dysfunction in TLR signalling may identify patients at high risk of post-operative SIRS and this is most likely mediated through the intermediate monocyte subpopulation, although monocyte subpopulations were not studied in these Phosflow experiments.

7. Accuracy of TLR signalling components as predictors of SIRS

It has been demonstrated that the markers of innate immune dysfunction identified in this study could be used as clinical markers of SIRS following major surgery. It was not possible to compare the experimental parameters presented in this thesis in a multivariate analysis because patients who developed SIRS could be separated 'perfectly' from those who did not. Interestingly, a multivariate analysis of routinely collected clinical parameters identified an independent association between SIRS and peripheral blood white cell count on post-operative day one (OR 0.49, p=0.03) after adjusting for age, sex, blood loss, receipt of blood products, CRP and type of operation (Table 7.1).

To assess the sensitivity and specificity of the innate immune markers evaluated in this thesis, we calculated the area under the receiver-operator curve (AUROC, albeit in this derivation set) for those tests that could be performed conveniently in a clinical pathology setting as predictors of SIRS (Figure 7.1). The AUROC, sensitivity, and specificity for IL-6, TLR4 and TLR5 expression on day 1 and day 2 were substantially greater than that for WCC on day 1, indicating greater accuracy for these tests compared to routine clinical parameters (Table 7.1).

Although this data indicates a significant improvement in sensitivity and specificity of TLR4, TLR5 and IL-6 over routinely used clinical parameters, we acknowledge that these findings require further evaluation in a larger, validation cohort.

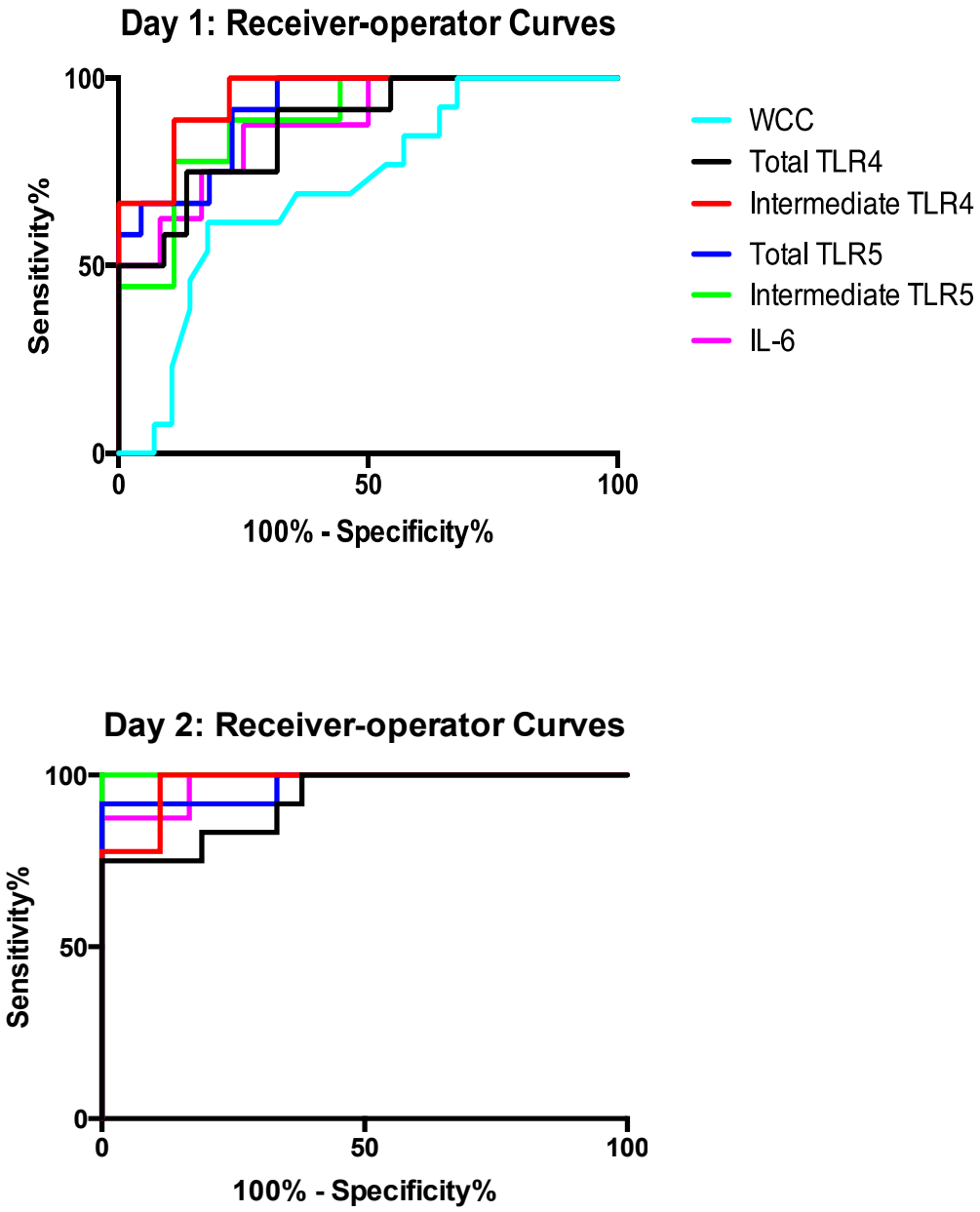


Figure 7.1. Receiver-operator curves for innate immune markers studied in this thesis compared to WCC on day one and day two following surgery (day two AUROC did not include WCC).

Parameter	AUROC	95% CI	P value	Cut off*	Sensitivity (%)	Specificity (%)
Day 1						
WCC	0.71	0.55 – 0.88	0.03	$13.35x10^3$/ ml	61.5	82.1
IL6	0.88	0.72 – 1.03	0.006	97.7 pg/ml	75	83
Total TLR4	0.87	0.75 – 0.99	0.0004	2000	75.0	68.2
Intermediate TLR4	0.95	0.86 – 1.04	0.001	8793	88.9	88.9
TLR5	0.92	0.83 – 1.00	<0.0001	2399	75.0	81.2
Intermediate TLR5	0.89	0.74 – 1.00	0.005	5437	88.9	77.8
Day 2						
IL-6				97.7 pg/ml	100	83.3
TLR4	0.93	0.83 – 1.02	<0.0001	2997	83.3	71.4
Intermediate TLR4	0.98	0.92 – 1.04	0.0007	9515	100	88.9
TLR5	0.97	0.92 – 1.03	<0.0001	3739	91.7	100
Intermediate TLR5	1.00	1.00 – 1.00	0.0003	7426	100	100

Table 7.1. Area under the receiver-operator curve for prediction of SIRS in patients undergoing complex HPB surgery. * mean fluorescence intensity units unless otherwise indicated.

8. General discussion

8.1 Study findings

As highlighted earlier, patients undergoing HPB surgery are at high risk of developing post-operative SIRS. The incidence of post-operative SIRS in this cohort was 31.7% and as stated in the introduction, inpatients who develop SIRS have a 7-fold increase in mortality compared to those who do not. The median length of time to the diagnosis of SIRS was 6 days following surgery and 92.3% (12/13) patients who were diagnosed with SIRS met the criteria for sepsis. The median length of hospital stay was 22 days in patients that developed SIRS and 13 days in those with an uneventful recovery and the mortality rate in the SIRS group was 15.3%. The results in this thesis have shown that patients who go on to develop post-operative SIRS might be identified even before surgery, in that those who develop SIRS have an increased proportion of intermediate monocytes and an exaggerated response to IFN2α induced STAT1 phosphorylation. Following surgery, monocyte TLR4 and TLR5 expression is significantly greater in patients who develop SIRS, specifically in intermediate monocytes. The increase in TLR expression was associated with altered transcription factor (NF-κB and ERK1/2) and cytokine production (IL-6).

Although SIRS increases the risk of morbidity and mortality in hospital patients, an inflammatory response to major surgery is not abnormal. It increases the antigen-sensing ability of the immune system and likely plays a significant role in preventing infections following surgery. However, problems arise when this inflammatory response to major surgery is dysregulated and SIRS develops. SIRS is part of the well described pathway that involves sepsis, severe sepsis and septic shock. In current clinical practice, systems such as the national early warning scoring system (NEWS) alert clinicians to patients at high risk of deterioration. Although simple to measure, NEWS most commonly identifies patients with established SIRS. The advent of novel innate immune biomarkers that predict patients at high risk of developing post-operative SIRS in the early post-operative period would allow

tailored care for patients before SIRS is clinically apparent and may therefore reduce post-operative complications.

In this thesis, I evaluated the role of monocyte TLR4 and TLR5 expression and key proteins in the signalling cascade in predicting post-operative SIRS. This has produced novel and exciting data and suggested a potential role for measuring TLR expression following surgery to identify high risk patients. However, all the experiments done in this thesis were performed ex-vivo. Although this does lend itself towards use as a potential test as a biomarker for SIRS if further validated, I cannot extrapolate the findings to an in-vivo model. This is due to more complex interactions between monocytes, macrophages, lymphocytes, endothelium and other organs involved in immune function.

To assess changes in transcription factor integral to the innate inflammatory cascade, I used multiplex phosflow. This was the first time in the literature that multiple transcription factors had been evaluated simultaneously in the same FACS tube using this technique. It provided the interesting finding of a concurrent increase in NF-κB and decreasing in ERK1/2 phosphorylation in patients who develop SIRS. Along with evaluating monocyte TLR4 and TLR5 expression, multiplex inflammatory transcription factor phosflow could have a multitude of uses. It could be used to identify patients at high risk of developing SIRS following all types of major surgery. In addition to this, it could potentially provide valuable information in inflammatory bowel disease, RA or SLE progression given the importance of abnormal inflammatory processes in these diseases.

8.2 Possible mechanism and future experiments

In patients who develop SIRS, circulating monocytes may be hyper-responsive to IFN. Increased STAT1 phosphorylation leads to the transcription and production of monocyte TLR4 and TLR5 which in turn increases in expression in response to major surgery. Increased

TLR expression allows greater TLR agonist binding which leads in turn to greater NF-κB phosphorylation and a concurrent decrease in ERK1/2 phosphorylation. This suggests preferential activation of NF-κB and subsequent pro-inflammatory cytokine production (Figure 8.1). These changes occur within 36 hours of major surgery. In addition to the findings in TLR signalling, patients who develop SIRS have a greater proportion of $CD14^{+}/CD16^{+}$ (intermediate) monocytes pre-operatively. These monocytes were found to have significantly greater TLR4 and TLR5 expression than CD14+/CD16- (classical) monocytes. It is therefore possible that intermediate monocytes are more responsive to IFN than other classical monocytes and the proportion of intermediate monocytes is greater in patients who develop SIRS. This indicates that although dysfunction in TLR signalling may identify patients at high risk of post-operative SIRS, this is most likely mediated through the intermediate monocyte subpopulation.

To evaluate whether the pathway proposed in Figure 8.1 was correct, a number of further experiments are required. The hypothesis that IFN stimulation of PBMCs leads to increased TLR4 and TLR5 mRNA production could be tested by stimulating PBMCs with IFN2α. Following stimulation, RNA could then be extracted to evaluate TLR4 and TLR5 mRNA expression using RT-PCR. Further to this, to test the hypothesis that type I IFNs play a substantial role in inflammatory cytokine production in these patients, recapitulation of previous experiments with the addition of an IFNAR1 blocking antibody would provide useful information on whether type I IFNs affects the production of inflammatory cytokines. If cytokine production were reduced following IFNAR1 blockade, this would implicate STAT1 as an important modulator of the maximal inflammatory response, and may explain why patients with elevated STAT1 phosphorylation pre-operatively have a greater cytokine response. If this hypothesis held true, it would suggest that low dose IFN blockade peri-operatively may reduce post-operative SIRS in susceptible patients. Given that changes were seen in TLR4 and TLR5 expression, it would also be of interest to look for changes in other TLRs, specifically TLR2. The agonists for TLR2 include lipoteichoic acid, a major constituent of gram-positive bacteria. On the same basis, a Luminex multiplex assay would allow assessment of multiple other cytokines. It would be of particular interest to look at changes in TLR agonist stimulated IL-1β and IL-8 concentration. In addition to assessing different

cytokines, changing the length of PBMC and TLR agonist incubation (e.g. 6 hour incubation) may also change the profile of cytokine elevation.

In addition to work aimed at further characterising the role of IFN and STAT1 in TLR expression and cytokine production, it is important to further delineate the role of intermediate monocytes in patients who develop SIRS. As described in this thesis and previously, intermediate ($CD14^{+}/CD16^{+}$) monocytes have greater TLR4 (222) and TLR5 expression than classical ($CD14^{+}/CD16^{-}$) monocytes. To test the hypothesis that intermediate monocytes produce more inflammatory cytokines than classical monocytes in this cohort of patients, intermediate monocytes could be removed from the heterogenous PBMC population by antibody specific techniques. These cells could then be stimulated with LPS and flagellin and the supernatants used for cytokine quantification using ELISA. In turn, NF-κB, ERK1/2 and STAT1 phosphorylation could be assessed in intermediate monocytes using multiplex phosflow. This would test the hypothesis that there is greater NF-κB phosphorylation in this subset of monocytes which in turn drives inflammatory cytokine production.

8.3 Validating the findings of this thesis

The data presented in this thesis are novel and the markers investigated have the potential to be used as a biomarker for post-operative SIRS. However, it is essential that the findings are validated. A larger study utilising the same innate immune assays in a more heterogenous surgical cohort (e.g. HPB, upper gastrointestinal, colorectal, vascular and transplant surgery) could identify whether monocyte TLR4 and TLR5 expression is elevated in all patients undergoing major abdominal surgery. This would be an important step in determining whether assessing TLR4 and TLR5 expression could be used to predict post-operative SIRS in a broader population. This approach would allow the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) to be calculated to determine the feasibility of using TLR4 and TLR5 expression as a biomarker of

post-operative SIRS. If a larger study confirmed these findings, blood could be taken on the morning phlebotomy round following surgery and monocyte TLR4 and TLR5 expression would be available later that day. If a patient was found to have elevated monocyte TLR4 and TLR5 expression and met a statistically derived 'cut-off', the post-operative management could be tailored in an attempt to prevent SIRS and tested in a randomised, controlled manner. There may be benefit in extending the HDU for more intensive monitoring. Antibiotics could be commenced empirically, given that almost all cases of SIRS resulted in infection. Granulocyte-macrophage colony-stimulating factor (GM-CSF) could be used to promote white cell production. One might even consider a short course of steroids (e.g. glucocorticoids, which inhibit NF-κB through induction of IκB synthesis), thereby inhibiting cytokine production (223), although the risks of immunosuppression may outweigh any anti-inflammatory benefit. Although the pharmacological agents required are not yet available, it is attractive to consider 're-setting' the innate immune response from a SIRS-prone to a non-SIRS phenotype by modulating STAT1 phosphorylation, TLR expression or signalling

Following a larger study to confirm the importance of TLR4 and TLR5 signalling as a marker for post-operative SIRS, the next step could involve an intervention with a novel TLR antagonist. This thesis has demonstrated that increased cytokine production following TLR ligand binding is signalled through preferential activation of NF-κB with a concurrent reduction in ERK1/2 phosphorylation. This opens up the possibility of evaluating TLR4 and TLR5 antagonists in attenuating the increased cytokine response in patients following major surgery. Mouse models have shown that TLR4 antagonists blocks influenza-induced lethality by reducing cytokine production and oxidised phospholipid expression (224). However, a phase 3 randomised control trial (RCT) found that eritoran (the same TLR4 antagonist used by Shirey et al.) did not reduce 28 day mortality in patients with severe sepsis compared to placebo (225). Patients with established severe sepsis have widespread dysfunction of multiple innate and adaptive immune responses; therefore inhibiting TLR4 alone is unlikely to attenuate the cytokine response to sepsis. Furthermore, it may be that sepsis is too late a stage in which to try to re-set TLR function, whereas access to an early marker of efficacy (such as TLR or TLR5 expression) may allow the drug to be used in only those patients who

will benefit. It would be interesting to perform a double-blinded RCT evaluating whether peri-operative eritoran could reduce post-operative SIRS compared to placebo, in. TLR5 antagonists have not been evaluated in the literature. Multiple NF-κB inhibitors have been identified, including commonly used medications (Melatonin, N-acetyl-cysteine, vitamin C, aspirin, losartan and glucocorticoids). These medications have been shown to be safe for use in humans but inhibition of NF-κB is potentially hazardous. NF-κB is a family of proteins that is ubiquitously expressed in almost all cells, so inhibition may result in adverse effects, although this may be alleviated by only giving a short peri-operative course of the medication. If the findings of this thesis are recapitulated in a larger study, evaluation of TLR signalling modulation in the peri-operative period following major surgery in a clinical trial may show a significant reduction in post-operative SIRS.

In conclusion, I have shown that innate immune dysfunction can be used to identify patients at high risk of developing SIRS following HPB surgery. Specifically, elevated monocyte TLR4 and TLR5 expression with preferential NF-κB phosphorylation led to increased cytokine production in patients who developed SIRS. Measurement of monocyte TLR expression could potentially be used as a biomarker of post-operative SIRS and allow tailored post-operative management of patients. Further to this, novel therapeutics such as peri-operative TLR antagonists may have the potential to reduce the incidence of SIRS following major surgery. A larger study including different general surgical subspecialties could validate these findings and precede an RCT evaluating a potential role for TLR or NF-κB antagonists in reducing post-operative SIRS.

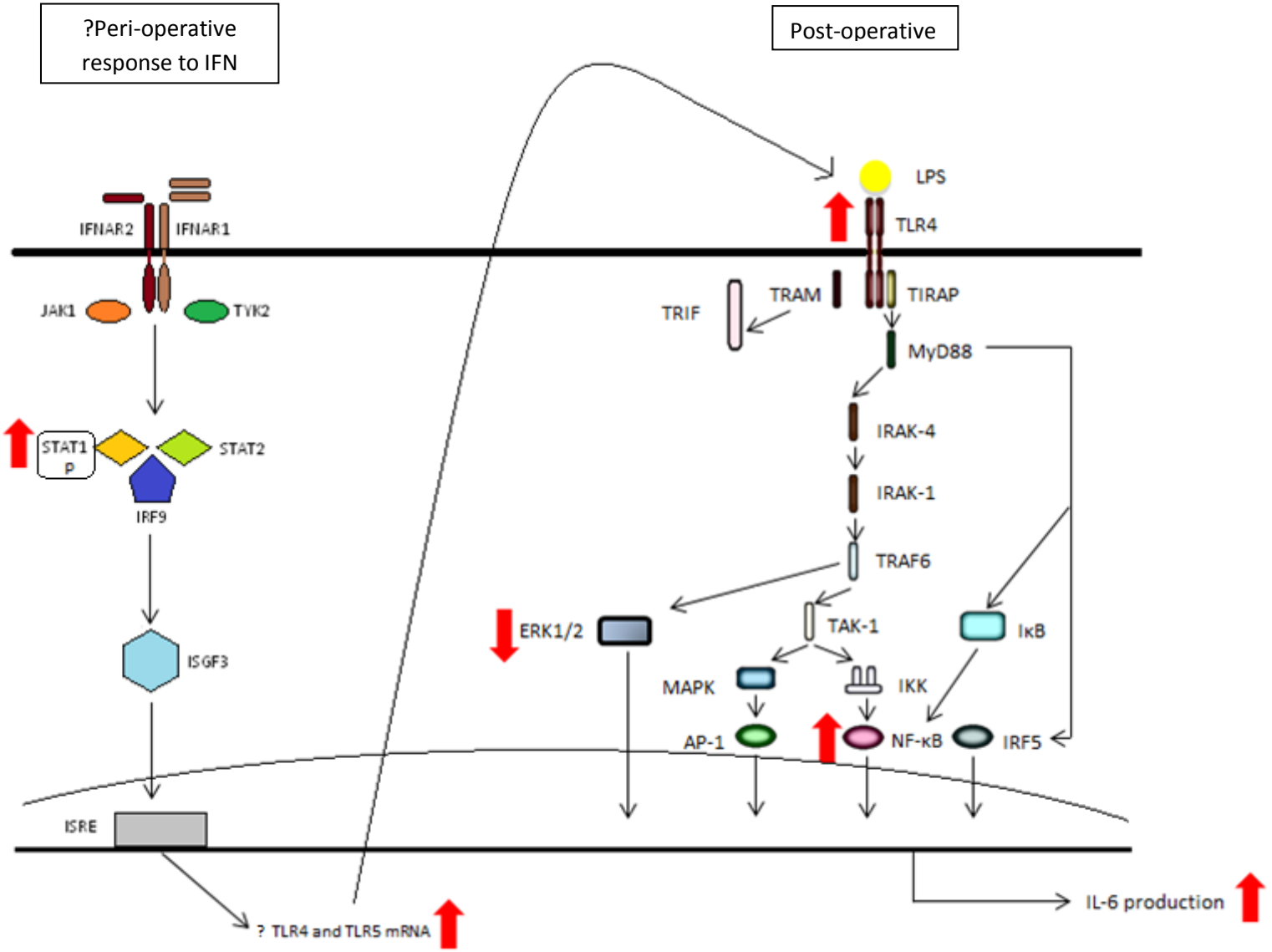


Figure 8.1. The possible pathway leading to increased cytokine production in patients who develop SIRS. Increased STAT1 phosphorylation (STAT1 p) in response to IFN2α leads to greater TLR4 and TLR5 mRNA transcription. Monocyte TLR expression is increased in response to surgery and leads to increased NF-κB and decreased ERK1/2 phosphorylation. This in turn leads to increased cytokine production

9. References

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10.1 Appendix 1.

Histological features of cohort

Patient number	Histology	TNM staging	Lymph node status
100	Fibromatosis	$T_0 N_0 M_0$	0/12
101	IPMN, no malignancy	$T_0 N_0 M_0$	0/15
102	Pancreatic ductal adenocarcinoma	$T_3 N_1 M_0$	4/11
103	Adenocarcinoma of distal CBD	$T_3 N_1 M_0$	14/20
104	Palliative bypass	n/a	n/a
105	Pancreatic ductal adenocarcinoma	$T_3 N_1 M_0$	2/15
106	Poorly differentiated ampullary adenocarcinoma	$T_2 N_1 M_0$	2/17
107	Poorly differentiated endocrine carcinoma arising from the duodenum	$T_3 N_1 M_0$	6/28
108	Moderately differentiated pancreatic adenocarcinoma. R1 resection	$T_3 N_1 M_0$	4/12
109	Poorly differentiated ampullary adenocarcinoma	$T_3 N_1 M_0$	5/12
110	IPMN, no malignancy	$T_0 N_0 M_0$	0/10
111	Benign stricture of the CBD. No underlying malignancy	$T_0 N_0 M_0$	0/18
112	Solid pseudo-papillary neoplasm. Tumour at posterior surface.	$T_3 N_0 M_0$	0/12
113	Poorly differentiated ductal adenocarcinoma	$T_3 N_1 M_0$	10/25
114	Grade 2 neuroendocrine tumour with vascular invasion.	$T_2 N_0 M_0$	0/16
115	Poorly differentiated ductal adenocarcinoma	$T_2 N_1 M_0$	3/21
116	Poorly differentiated ductal adenocarcinoma	$T_3 N_1 M_0$	1/18
117	No viable tumour in specimen. No evidence of malignancy.	n/a	0/0
118	Moderately differentiated adenocarcinoma with morphology in keeping with colorectal primary. Complete excision	n/a	0/0
119	Chronic cholecystitis and	n/a	0/0

	choledochal cyst. No dysplasia or malignancy		
120	Right hemi-hepatectomy specimen - no viable tumour. Wedge resection - metastatic adenocarcinoma consistent with colorectal primary	n/a	0/0
121	Segment 5 & 6 - single metastatic colorectal adenocarcinoma metastasis excised by 0.1mm	n/a	0/0
122	Low grade tubular adenoma of the duodenum. No evidence of malignancy.	$T_0 N_0 M_0$	0/14
123	IPMN with low grade dysplasia	$T_0 N_0 M_0$	0/11
124	Metastatic renal cell carcinoma (clear cell). No necrosis. Tumour at SMV margin	$T_3 N_1 M_0$	3/12
125	IPMN with low grade dysplasia	$T_0 N_0 M_0$	0/20
126	Mucinous cystadenoma at the tail of the pancreas. No malignancy	$T_0 N_0 M_0$	0/18
127	Poorly differentiated pancreatic ductal adenocarcinoma	$T_3 N_1 M_0$	3/11
128	8mm benign neuroendocrine tumour	$T_1 N_0 M_x$	0/9
129	Poorly differentiated pancreatic ductal adenocarcinoma. Tumour at uncinate process margin.	$T_3 N_1 M_0$	3/8
130	Tubovillous adenoma with low grade dysplasia. Lymph node negative. No malignancy	$T_0 N_0 M_0$	0/11
131	Metastatic neuroendocrine tumour. Positive margin.	n/a	n/a
132	Poorly differentiated ampullary adenocarcinoma. Pancreatic infiltration.	$T_4 N_1 M_0$	2/25
133	Ulcerated duodenal malignant melanoma. Vascular invasion.	$T_4 N_1 M_0$	2/16
134	Benign microcystic serous cystadenoma. No malignancy.	$T_0 N_0 M_0$	0/22
135	Completely excised metastatic adenocarcinoma	n/a	3/4
136	Palliative bypass	n/a	n/a
137	Segment 8 metastatic adenocarcinoma. Margin involved	n/a	n/a

138	Moderately differentiated ductal adenocarcinoma	T_3 N_1 M_0	1/18

The histological features of the study cohort following resection. The table includes pathological TNM staging.

10.2 Appendix 2.

Manuscript submitted to Science Translational Medicine

Systemic inflammatory response syndrome (SIRS) after major abdominal surgery is predicted by early activation of TLR5

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Post-operative activation of TLR5 predicts systemic inflammatory response syndrome in patients recovering from major abdominal surgery

(135c + spaces)

Abstract

Patients undergoing major surgery are at risk of life-threatening complications including systemic inflammatory response syndrome (SIRS) and sepsis. Early identification of patients at risk of SIRS would allow tailored post-operative care and improve survival. Serum IL-6 levels are upregulated in patients who exhibit poor outcomes after surgery, but the mechanisms underlying this response are currently unknown. We studied these mechanisms in patients undergoing complex abdominal surgery to identify early biomarkers of altered inflammatory responses and poor clinical outcomes.

Serum concentrations of IL-6 on postoperative Day 2 were significantly increased in 12 patients who developed SIRS (median postoperative day 6) compared with 27 patients who did not. Peripheral blood mononuclear cells (PBMCs) sampled from SIRS patients on Day 1 and Day 2 after surgery (prior to clinical signs of SIRS) displayed significantly greater TLR4 and TLR5 expression and produced significantly more IL-6 in response to LPS and flagellin. Consistent with these data, TLR-driven phosphorylation of NF-κB (but not ERK1/2) was increased on Day 1, and interferon alpha-mediated STAT1 phosphorylation was higher pre-operatively in SIRS patients.

Increased TLR4 and TLR5 expression was confirmed in an independent patient cohort. Differences in TLR4 and TLR5 expression were greatest in the $CD14^{++}CD16^{+}$ 'intermediate' monocyte population. Intermediate monocyte TLR4/5 expression on Day 1 or Day 2 predicted SIRS development with an accuracy of 0.89 - 1.0 (calculated areas under the receiver operator curves).

Markers of innate immune dysfunction can be used 5 days before the onset of clinical signs to identify patients at risk of SIRS.

Introduction

The systemic inflammatory response syndrome (SIRS) is associated with significant patient morbidity and mortality after major surgery[1]. SIRS is a clinically-defined state that represents activation of inflammatory, innate immune, coagulation and repair pathways and is frequently observed in hospitalised patients. Overall, there is an almost 7-fold increase in 28 day mortality in hospitalised patients with SIRS compared to those without[4]. The incidence of post-operative sepsis (SIRS plus presumed or confirmed infection) is high in major operations; 16.7% following distal pancreatic resections[2], or 32-46% in digestive and gynaecological tumour resections[3]. Morbidity (including sepsis and anastomotic leak) following pancreaticoduodenectomy ranges from 30-45%[5,6] and 30 day mortality is almost 4%.[5,7,8]. Pre-emptive therapy cannot be given to patients at risk of developing SIRS because clinically-useful peri-operative predictive factors have not yet been identified.

A key step in the initiation of SIRS is activation of toll-like receptors (TLRs) by pathogen associate molecular patterns (PAMPs)[9]. The paradigm for this family of receptors is TLR4 interacting with LPS, a component of gram-negative bacteria. There are at least 11 other members of the TLR family in humans and each is associated with activation by particular PAMPs[10]. TLR5 is activated by flagellin, which is a component of motile bacteria[11]. Following receptor ligation, adapter proteins are recruited and intracellular signalling pathways eventually lead to the activation (phosphorylation) of key transcription factors such as NF-□B and ERK1/2 amongst others[12, 13]. These in turn drive the production of important pro- and anti-inflammatory cytokines such as IL-6, TNF-□ and IL-10[14].

Following surgery, dysregulated release of pro-inflammatory cytokines has been associated with post-operative complications. Many groups have attempted to identify biomarkers that predict poor outcomes following surgery, but the majority of these tests are inconvenient to run in a clinical setting or involve unwieldy or expensive techniques[15,16]. Despite this complexity, several reports have suggested that IL-6 concentration in the early

post-operative period is a good predictor of post-operative SIRS[3,17]. Therefore, our aim was to study the pathways involved in IL-6 production in patients at high risk of SIRS with the goal of identifying early biomarkers of an enhanced inflammatory response that may lead to clinical consequences.

Methods

Patient selection

We included consenting adult patients who were undergoing major liver or pancreatic (non-emergency) resection at Barts Health NHS Trust between August 2011 and November 2013. Exclusion criteria were active concurrent inflammatory disease (e.g. inflammatory bowel disease, rheumatoid arthritis), pre-operative sepsis and treatment with chemotherapeutic or regular anti-inflammatory medications. Every patient on a weekday elective operating list was screened. Eligible patients were then approached for written informed consent.

Data collection

Demographic, clinical, haematological and histological data were collected prospectively from patient notes and the Electronic Patient Record (EPR) system and included information on co-morbidities, indication for surgery, cancer diagnosis, duration of the procedure, length of hospital stay and in-hospital mortality. Patients were examined daily by the clinical team for the presence of SIRS or infection. Definitions of infection were agreed *a priori* by the investigators and were based on the Center for Disease Control and Prevention definitions and graded using the Clavien-Dindo classification [2].

Venous blood sampling and separation

Bloods were taken at clinically-indicated times pre-operatively, and on day 1 and day 2 post-operatively. A total of 5ml was collected into serum-separating tubes and centrifuged at 600g for 10min. Serum was collected and stored at -80°C. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficoll Paque (GE Healthcare, Little Chalfont, UK) to provide a source of monocytes. The resulting interface was aspirated, washed in PBS and stored in10% DMSO freezing solution at -80°C.

TLR stimulation and cytokine production

A total of 2 x 10^6 PBMCs were re-suspended in 1ml complete medium (RPMI 1640 containing fetal calf serum (FCS), penicillin and streptomycin), then rested for two hours and equally divided into a 12 well plate. Each well contained 1ml complete medium per well. The cells were stimulated with 2ng/ml *S. typhi* lipopolysaccharide (LPS, Sigma, Gillingham, UK) and 10µg/ml *S. Typhi* flagellin (Invivogen, Toulouse, France) for 24h. Supernatants were stored at -80°C. IL-6, TNF-α and IL-10 concentrations were measured in thawed supernatants and patient serum samples using commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, USA).

Measurement of TLR4 and TLR5 cell surface expression on monocytes

Thawed PBMCs were incubated at room temperature in the dark for 45min with a combination of antibodies specific for the following: CD14 (Pacific Blue), CD16 (AlexaFluor 647®), TLR4 (Phycoerythrin (PE)) (Biolegend, Cambridge, UK) and TLR5 (Fluorscein isothiocyanate (FITC)) (Abcam, Cambridge, UK). Isotype-matched controls were obtained from the same manufacturers. Cells were fixed in 1% paraformaldehyde (PFA) and analysed immediately using a Beckton Dickinson Canto II flow cytometer. TLR4 and TLR5 expression was calculated and expressed by median fluorescence intensity (MFI), using Beckton Dickinson FACS Diva software.

Quantification of NF-□B, ERK1/2 and STAT1 phosphorylation in CD14 positive cells (Multiplex PhosFlow)

PBMCs were prepared as for TLR stimulation above and the contents of each well of a 6 well plate were re-suspended in 200µlf complete medium. Following incubation, the cells were aspirated from each well and transferred into FACS tubes. Each well was washed with 1ml complete medium to ensure complete removal of any cells that had adhered to the base of the well. There were 6 labelled tubes for each sample of PBMCs, these were labelled as; unstimulated isotype control, Unstimulated, Flagellin stimulated, LPS stimulated, interferon-

2α (IFN2α) stimulated and PMA stimulated. The samples were then centrifuged at 300g for 5min and the cells re-suspended in complete medium.

The following were added to the PBMCs in FACS tubes and incubated for 15min in a water bath at 37°C; 10µg/ml flagellin, 2ng/ml LPS, 10,000u/ml IFN2α, or no agonist. Cells were fixed in 1% PFA, permeabilized with Perm buffer III (BD Biosciences, San Jose, USA) on ice in the dark for 30min. PBMCs were then washed in FACS buffer (500ml PBS, 10ml fetal calf serum (FCS), 0.1g sodium azide, 0.18g EDTA) and the antibody cocktail added: anti-STAT1 (Fluorscein isothiocyanate (FITC)), anti-NF-kB (Phycoerythrin (PE)), anti-ERK1/2 (Alexa Fluor 647 (AF647)) (BD Biosciences, Oxford, UK) and anti-CD14 (Pacific Blue) (Biolegend, Cambridge, UK). The PBMCs were incubated at room temperature for 45min in the dark, washed with FACS buffer and re-suspended in 300µl FACS buffer and immediately analysed on a Canto II flow cytometer. Gating and compensation were performed to select $CD14^{+}$ cells for analysis of NF-kB, STAT1 and ERK1/2 phosphorylation. Each experiment was performed with an isotype control, as listed above. The data were analysed using WinList™ 3D version X.Y. (Verity Software House) and expressed as the percentage increase in phosphorylation above background.

Serum conditioning of healthy control PBMCs

PBMCs from healthy controls were prepared in the same way as patient samples. In serum conditioning experiments, 500µl patient serum was added to each well of a 12-well plate prior to stimulation with the same agonists as above. Supernatants were stored at -20°C until analysis by ELISA. PBMCs were then used for flow cytometry as described above.

Gene Expression

Blood was collected at each time point in a PAXGene^{TM} blood RNA tube (PreAnalytix, Hilden, Germany). Total RNA was extracted using PAXGene^{TM} blood RNA kits (PreAnalytix). Samples were analysed for RNA integrity and reverse transcribed to complementary DNA (cDNA) [3]. Gene expression was quantified by qRT-PCR using TLR4 and TLR5 Taqman assays (Applied

Biosystems, Foster City, CA) which spanned the final two exons of the most common isoform of each gene, and were carried out on a 7900HT apparatus, Life Tech (Applied Biosystems, Foster City, CA) as previously described [3]. Each sample was assayed in triplicate. Reference genes (β2 microglobulin (*B2M*) and Ubiquitin C (*UBC*)) were selected empirically from a panel of six [4]. Relative quantification was calculated using the standard delta-delta methodology. Results were expressed as a normalized ratio of candidate gene / reference gene.

Statistical analysis

All data presented in the results section were tested for normality using the D'Agostino Pearson test. Continuous data were analysed using appropriate statistical tests (unpaired t test, Mann Whitney test) based on normality of distribution. Differences in categorical variables were calculated using a chi-squared or Fisher's exact test as appropriate. All statistical analysis was performed using GraphPad Prism and $p<0.05$ was considered significant.

Results

Serum IL-6 concentrations predict post-surgical SIRS

Systemic inflammatory response syndrome (SIRS) after major surgery is associated with significant patient mortality, but the immune mechanisms that drive this pathology are not completely understood, and robust biomarkers of SIRS risk have yet to be defined. We therefore sought to identify immune parameters associated with the development of SIRS in a cohort of 39 patients undergoing 'high risk' surgical procedures (Table 1). Mean patient age was 63.2 years (range 22-85), 52% (n=20) were male, and 95% (n=37) were Caucasian (Table 1). A total of 24 patients underwent pancreaticoduodenectomy, 9 major liver resection, 4 distal pancreatectomy, 1 palliative bypass procedure and 1 excision of choledochal cyst. Of these 39 patients, 12 (30.7%) subsequently developed SIRS between post-operative days 2 and 12 (median day 6). The cause of each complication and the outcome are shown in Supplementary Table 1. The median length of hospital stay for patients who developed post-operative SIRS was significantly longer than for those who had an uneventful recovery (22 versus 12 days, $p<0.001$). All patients were managed with the same anaesthetic regime, and there were no differences in SIRS rates among the surgeons (data not shown). There were no significant differences in intraoperative transfusion of blood products or key clinical parameters (haemoglobin, white cell count, creatinine, bilirubin or C-reactive protein (CRP)) between patients who developed SIRS and those who did not (Table 1). There were also no significant differences in operative time and blood loss volume, except in 6 patients who underwent right hemi-hepatectomy, which is a complex procedure with longer duration (295min vs 198min, $p<0.05$) and greater blood loss (2695ml vs 1344ml, $p<0.01$).

A key step in the initiation of SIRS is activation of toll-like receptors (TLRs) by pathogen and damage associate molecular patterns (PAMPs and DAMPs)[9] leading to production of potent pro-inflammatory cytokines including IL-6. Accordingly, serum concentrations of IL-6 were higher in patients who developed SIRS after surgery compared with those who did not (Figure 1, 122.4pg/ml vs 92.1pg/ml on day 2, $p<0.05$). These changes in IL-6 cytokine level pre-dated changes in any of the other parameters assessed here. Post-operative serum

concentrations of the anti-inflammatory cytokine IL-10 were reduced in both groups of patients compared to preoperative levels (211.5 pg/ml pre-operatively vs 151.1 pg/ml on day 2, $p<0.02$), but there were no significant differences between groups. There were no changes in serum TNF concentration.

Monocyte TLR expression is associated with post-operative SIRS

Ligation of microbial products by TLRs, including TLR4 and TLR5, leads to rapid production of pro-inflammatory cytokines that have been implicated in the pathogenesis of SIRS. Since monocytes are a major TLR4-expressing cell type in human blood, we tested the hypothesis that monocytes are a major source of IL-6 cytokine in patients who develop SIRS. Expression levels of TLR4 and TLR5 on $CD14^+$ monocytes sampled on post-operative day 1 were 2.1-fold and 2.4-fold greater respectively in patients who developed post-operative SIRS than in those with an uneventful recovery (Figure 2, $p<0.0001$). Similar results were seen on post-operative day 2, with 2.1-fold and 2.7-fold higher TLR4 and TLR5 levels respectively in patients who developed SIRS. There were no significant pre-operative differences in TLR4 or in TLR5 expression between the two groups of patients, and this level of TLR expression in patients did not differ significantly from that of TLR expression in monocytes from healthy control volunteers. In patients who did not develop post-operative SIRS, there was no significant change in monocyte TLR4 and TLR5 expression in the peri-operative period. However, in the SIRS group, there was a significant increase in expression of TLR4 ($p<0.0002$) and TLR5 ($p<0.0001$) early after surgery (post-operative days 1 and 2; Supplementary Figure 1).

Consistent with these data, on post-operative day 2 (but not pre-operatively or on day 1), monocytes from patients who developed SIRS produced significantly more IL-6 following *ex vivo* stimulation with the TLR4 agonist LPS ($p<0.0001$) or TLR5 agonist flagellin ($p<0.0001$) when compared with monocytes from patients who did not develop SIRS (Figure 3 and Supplementary Figure 2). This was not the case for TLR-mediated TNF-α or IL-10 production (Figure 3). Interestingly, the tonic production of TNF-α by unstimulated monocytes was

significantly reduced in the immediate post-operative period compared with pre-operatively (pre-operative mean 200pg/ml, 109pg/ml on day 1, 91pg/ml on day 2, $p<0.0001$ ANOVA). In contrast, IL-6 or IL-10 production by unstimulated monocytes did not differ between pre-operative and early post-operative samples.

These findings were not due to the effects of stable circulating soluble mediators (including IL-6 itself), since changes in TLR4 or TLR5 expression and ligand-stimulated cytokine production could not be replicated by culturing blood monocytes from healthy controls in patient serum, regardless of whether or not the serum was derived from a SIRS patient (Supplementary Figures 3). Following LPS and flagellin stimulation, there was no significant difference in IL-6, TNF-α or IL-10 production between monocytes treated with sera from patients who develop SIRS or patients who did not (Supplementary Figure 4). These data suggest that the inflammatory abnormalities in patients who develop SIRS are intrinsic to monocytes and not to stable circulating factors.

We next sought to determine whether this intrinsic enhancement of monocyte TLR activation and cytokine production was associated with activation of canonical signalling pathways. To do this, we developed a multiplex assay based on the BD Bioscience PhosFlow system to study the phosphorylation of the key signalling molecules NF-κB, ERK1/2 and STAT1 (Figure 4). In-line with our *ex vivo* cytokine production data, monocyte stimulation with LPS or flagellin resulted in greater NF-κB phosphorylation in patients who developed SIRS compared with patients who did not on post-operative day 1 and day 2. LPS stimulation resulted in a 3.7-fold increase in NF-κB phosphorylation on day 1 compared with non-SIRS patients ($p<0.01$) and 11.2-fold increase on day 2 ($p<0.005$). Flagellin stimulation resulted in a 3.8 fold increase in NF-κB phosphorylation on day 1 compared with non-SIRS patients ($p<0.05$) and an 8.9 fold increase on day 2 ($p<0.005$).

ERK1/2 phosphorylation in blood monocytes was consistently lower in patients who developed SIRS than in patients who did not when assessed in unstimulated ($p<0.04$), LPS-stimulated ($p<0.03$) or flagellin-stimulated cells ($p<0.03$) as assessed on post-operative day 2. A similar trend was observed in monocytes from day 1, but these comparisons did not reach statistical significance.

There were no pre-operative differences in unstimulated, LPS- or flagellin-induced NF-κB or ERK 1/2 phosphorylation. However, pre-operative induction of STAT1 phosphorylation by IFNα cytokine (but not LPS or flagellin) was 1.9 fold greater in monocytes from patients who developed SIRS compared with those who did not ($p<0.05$).

Taken together, these data suggest that patients who proceed to develop SIRS exhibit increased TLR4 and TLR5 expression on blood monocytes in the period immediately after surgery. The functional consequence of this is increased activation of the NF-κB-IL-6 signalling pathway. The strong positive correlations between TLR expression, agonist-induced IL-6 production and NF-κB activation suggest that these phenomena are linked *in vivo* (Supplementary Figure 2).

Elevated TLR expression on intermediate monocytes from patients who develop SIRS

In order to determine whether the increased expression of TLR4 or TLR5 on blood monocytes was present on all subsets, or whether this was restricted to a particular sub-population, we studied CD16 expression to distinguish classical ($CD14^{++}$,$CD16^{-}$) intermediate ($CD14^{++}$,$CD16^{+}$) and non-classical ($CD14^{+}$,$CD16^{++}$) monocytes (Supplementary Figure 5). Pre-operatively, the proportions of intermediate monocytes were higher ($p<0.01$, Figure 5) and those of classical monocytes lower ($p<0.05$) in patients who developed SIRS compared to those who did not, but there were no significant post-operative differences in these proportions.

In all patients analysed here, monocyte expression of TLR4 ($p<0.0001$) and TLR5 ($p<0.0001$) were highest in the intermediate subset, and the increases in TLR4 and TLR5 levels seen in patients who proceeded to develop SIRS were restricted to this subset (Supplementary Figure 6). In keeping with the data from total monocytes, there were no pre-operative differences in TLR4 or TLR5 expression in any subset. However, on both post-operative days 1 and 2, TLR4 ($p<0.0004$) and TLR5 ($p<0.0001$) expression on intermediate monocytes was significantly higher in patients who developed SIRS compared with patients who had an uneventful recovery (Figure 5). On day 2, TLR4 and TLR5 expression levels were 1.5-fold higher in patients who developed SIRS compared with those who did not. Thus, TLR4 and TLR5 expression on intermediate monocytes progressively increased over time in patients who developed SIRS, whereas they remained constant in patients who exhibited a non-eventful recovery.

TLR5 expression predicts SIRS

We hypothesised that the markers of innate immune dysfunction identified in this study could be used as clinical markers of post-operative SIRS. It was not possible to compare the experimental parameters presented in the current manuscript in a multivariate analysis because patients who developed SIRS could be separated 'perfectly' from those who did not. We calculated the area under the receiver-operator curve (AUROC, Figure 6) to estimate the accuracy of those tests that could be performed conveniently in a clinical pathology setting as predictors of SIRS. The AUROC, sensitivity, and specificity for IL-6, TLR4 and TLR5 expression on day 1 and day 2 were substantially greater than that for WCC on day 1, indicating greater accuracy for these tests compared to routine clinical parameters (Table 2).

We next sought to confirm that increased monocyte TLR expression in the early post-operative period was indeed associated with SIRS by studying whole blood gene expression in a second cohort (Cohort B) of 31 different patients recruited from the same hospital with the same inclusion and exclusion criteria. The baseline demographic and

clinical characteristics of patients in cohort B were similar to those of the main group with no significant differences in sex, age, ethnicity, intraoperative time, receipt of intraoperative blood products, pre-operative biochemistry and haematology (Table 3). In Cohort B, 8 patients (25.8%) developed SIRS in the post-operative period, all associated with the subsequent development of infection. As in the original cohort, patients who developed SIRS had a significantly longer in-patient stay (median 22 vs 9 days, $p<0.001$). Cohort B patients who developed SIRS also had significantly greater IL-6 concentration in the serum (233.9 pg/ml vs 71 pg/ml, $p=0.002$) on day 2 but this did not reach statistical significance on day 1. TLR5 gene expression increased significantly post-operatively compared to pre-operative samples in this cohort. In keeping with the findings from our earlier protein-based studies in selected monocytes, the expression of TLR5 mRNA in whole blood was 35% greater in patients who went on to develop SIRS compared to those who did not (Figure 7, $p<0.01$).

Discussion

SIRS is a common complication of major surgery and identifying patients who will develop SIRS early in the post-operative period is an important clinical goal. Here we report that serum concentration of IL-6 cytokine on post-operative days 1 and 2 can be used to distinguish patients who will develop SIRS from those who will not - a median of 5 days before the onset of clinical signs in our series. We have studied the mechanisms that drive IL-6 production and show that in patients who develop post-operative SIRS, both LPS and flagellin can drive monocyte IL-6 production to a greater extent than in patients who do not, and this is likely to be mediated by upregulated TLR gene expression, as indicated by mRNA Taqman PCR in whole blood from patients in an independent cohort. As such, we have identified TLR4 and TLR5 expression on monocytes, especially intermediate monocytes, as early and accurate biomarkers for post-operative SIRS.

IL-6 is a pleotropic inflammatory cytokine that forms an important part of the early response to injury or infection. It activates vascular endothelial cells increasing permeability and vascular leakage, stimulates the hepatic production of acute phase proteins (including C-reactive protein), promotes bone marrow production of inflammatory and immune cells, and can activate B and T lymphocytes[18]. We and others have observed that IL-6 is elevated in patients who develop SIRS, and although IL-6 concentrations were lower in our series than those reported by studies of similar patient groups[3], it is a consistent finding that IL-6 levels on day 1 can distinguish patients who will develop SIRS from those who will not[17]. We further investigated the mechanisms underlying these data, and detected that the TLR/NF-κB/IL-6 axis is over-active in patients who develop SIRS. While responses to both TLR4 and TLR5 agonists were increased, the abnormality is specific to the NF-κB but not ERK1/2 signalling, and only IL-6 production was elevated (not TNF or IL-10), suggesting that additional intracellular factors determine selectivity of response. Activation of NF-κB in CD14+ monocytes has recently been shown to be associated with poor recovery following orthopaedic surgery (recovery from fatigue, functional impairment of the hip, and pain after surgery), although this study did not explore the mechanisms or pathways that result in transcription factor activation.

We have focussed on key TLRs that mediate host responses to bacterial products, although the source of these products is as yet unclear, and the role of additional PAMPs and TLRs (e.g. lipoteichoic acid ligation of TLR2) is currently unknown. We postulate that abdominal surgery, and manipulation of the gut in particular, results in translocation of gut-derived microbiota into the circulation, although other sources of bacteria, other TLR agonists, and DAMPs will require future evaluation as potential triggers of inflammation.

TLR-mediated production of type 1 IFN (IFN□/□□ is a very early step in the initiation of inflammation. Autocrine and paracrine type 1 IFN binds to the cognate receptor and activates signalling mediators including STAT1 and STAT2, which in-turn drive IRF-mediated gene expression and amplify the response[19]. It is therefore of particular interest to note pre-operative differences in IFNα-induced STAT1 phosphorylation between patients who develop SIRS and those who do not. Our hypothesis is that individuals who develop SIRS are pre-disposed to excessive inflammation with a 'primed' STAT1 response. For a given degree of tissue damage, endogenous bacterial translocation (or an alternative stimulus), this 'pre-primed' state facilitates a more robust inflammatory response, which may include expression of TLRs (known IFN stimulated genes). In keeping with this, we have shown an increased abundance of TLR5 mRNA in patients who develop SIRS. However we do not currently know which mediator is responsible for this priming, although inhibitors of TLR and IFN signalling – such as SOCS and PIAS proteins are potential candidates.

These insights into the putative mechanism for the development of SIRS are possible because we have developed multiplex Phosflow to enable the simultaneous assessment of transcription factor activation in CD14+ monocytes in a single experimental assay. It would be interesting to see if the kinetics of transcription factor activation also differ in patients who develop SIRS and those who do not.

Patients who develop SIRS have a greater proportion of $CD14^{++}/CD16^{+}$ (intermediate) monocytes pre-operatively. Intermediate monocytes are considered pro-inflammatory with greater production of TNF-□ and IL-1□ following stimulation with LPS, increased interaction with endothelial cells and greater chemokine production compared to other subsets[20]. We show that intermediate monocytes express higher levels of TLR4[21] and TLR5 (previously not known) than $CD14^{+}/CD16^{-}$ classical or $CD14^{+}/CD16^{++}$ non-classical monocytes at all time points. The increased expression of TLR4 and TLR5 detected on monocytes from patients who develop SIRS was restricted to the intermediate subset, and accordingly, the accuracy of predicting SIRS is increased by measuring TLR4 and TLR5 on intermediate monocytes only (compared to total monocytes).

The incidence of post-operative SIRS in our main cohort was 31.7%, and SIRS was associated with a significantly increased length of hospital stay (22 days versus 13 days) and a higher mortality rate (15.3%). Despite the heterogeneity of the operations included in this study, these outcomes are in keeping with published data[5,6] suggesting that our conclusions are applicable more widely. The diagnostic accuracy of the tests proposed in this study will now require validation in a larger, independent cohort, and it may be possible to refine our protocols yet further to enable these tests to be conducted in a routine clinical setting.

Currently-used 'early warning' scoring systems are based on routinely collected clinical data, but these can be difficult to interpret in post-operative patients in whom clinical observations may already be deranged. These scores are therefore unable to identify patients at risk until SIRS is established, or nearly established. Our current experience suggests that this is too late to implement preventative measures such as low-dose glucocorticoids, antibiotics and/or prolonged stay on the high-dependency unit. In contrast, the novel approach that we propose may permit the use of interventions that are targeted against key inflammatory mediators of SIRS pathogenesis early after surgery. Although targeted therapies such as specific TLR4 inhibitors did not alter outcomes in patients with

severe sepsis (221) (222), this may be due to the fact that patients were already critically unwell and at advanced stages of SIRS when given a TLR4 antagonist. We hypothesise that treating patients with TLR4 inhibitors or agents that impair signalling through TLR5 or NF-kB may provide therapeutic benefit before the clinical manifestations of SIRS or sepsis become apparent.

In conclusion, we have identified a novel mechanism by which innate immune dysfunction occurs early in the post-operative period in patients who develop SIRS – long in advance of clinical signs. Abnormally elevated IL-6 levels in patients who will develop SIRS are related to upregulated TLR4 and TLR5 expression and preferential activation of NF-kB. We hypothesise that prior to surgery, patients who develop SIRS exhibit an increased proportion of intermediate monocytes that are primed to react in this way. Clearly more work is needed to develop the clinical application of the markers we have identified here. However, we believe that the strategy we have employed here to study the mechanisms of disease have identified novel biomarkers as well as potential targets for therapeutic intervention.

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Table 1. Demographic and clinical parameters.

	No SIRS		SIRS		P value
Sex	Male 6/20	Female 7/19	Male 14/20	Female 12/19	p=ns
Ethnicity	Caucasian 25/37	Other 1/2	Caucasian 12/37	Other 1/2	p=ns
Operative Factors					
Operative time (mins)	270 (135-460)		323 (170-585)		p=ns
Blood loss (mls)	773 (190-2010)		1088 (150-3200)		p=ns
Intra-operative transfusion (n(%))	7 (26)		6 (50)		p=ns
Pre-operative values					
Hb	13.1 (12-14.5)		12.8 (11-14.5)		p=ns
WCC	8.1 (5.2-10.4)		7.9 (5.2-10)		p=ns
Plts	274 (138-411)		265 (129-402)		p=ns
Ur	5.1 (3.5-8.4)		4.8 (3.8-8.6)		p=ns
Cr	79 (59-102)		81 (48-147)		p=ns
Bili	18 (0-48)		12 (0-31)		p=ns
Post-operative day one					
Hb	10.1 (8.6-12.5)		10.2 (9.0-12.5)		p=ns
WCC	13.1 (9.2-15.1)		14.1 (8.0-17.9)		p=ns
Plts	221 (104-442)		210 (98-429)		p=ns
Ur	6.6 (4.4-10.2)		6.9 (3.9-10.5)		
Cr	75 (49-100)		81 (41-143)		p=ns
Bili	18 (3-48)		15 (8-30)		p=ns
CRP	49 (21-76)		52 (5-108)		p=ns
Post-operative day two					
Hb	9.6 (7.9-13.0)		9.4 (7.8-12.8)		p=ns
WCC	14.1 (7.5-20.3)		14.5 (7.8-24.1)		p=ns
Plts	202 (99-400)		198 (83-389)		p=ns
Ur	6.3 (3.4-8.8)		6.2 (3.3-8.5)		p=ns
Cr	81 (48-167)		78 (41-150)		p=ns
Bili	14 (4-31)		15 (4-46)		p=ns
CRP	111 (23-203)		118 (48-257)		p=ns

Parameter	AUROC	95% CI	P value	Cut off*	Sensitivity (%)	Specificity (%)
Day 1						
WCC	0.71	0.55 – 0.88	0.03	$13.35x10^3$/ ml	61.5	82.1
IL6	0.88	0.72 – 1.03	0.006	97.7 pg/ml	75	83
Total TLR4	0.87	0.75 – 0.99	0.0004	2000	75.0	68.2
Intermediate TLR4	0.95	0.86 – 1.04	0.001	8793	88.9	88.9
TLR5	0.92	0.83 – 1.00	<0.0001	2399	75.0	81.2
Intermediate TLR5	0.89	0.74 – 1.00	0.005	5437	88.9	77.8
Day 2						
IL-6	0.98	0.93-1.03	0.0004	97.7 pg/ml	100	83.3
TLR4	0.93	0.83 – 1.02	<0.0001	2997	83.3	71.4
Intermediate TLR4	0.98	0.92 – 1.04	0.0007	9515	100	88.9
TLR5	0.97	0.92 – 1.03	<0.0001	3739	91.7	100
Intermediate TLR5	1.00	1.00 – 1.00	0.0003	7426	100	100

Table 2: Area under the receiver-operator curve for prediction of SIRS in patients undergoing complex HPB surgery. * mean fluorescence intensity units unless otherwise indicated.

Operation	Day SIRS/Sepsis criteria met	Cause	Outcome	Discharge
PPPD	POD 6	Lower respiratory tract infection	IV antibiotics and transferred to HDU for positive pressure ventilation	POD 27
PPPD	POD 8	Infected intra-abdominal collection	IV antibiotics. Did not require drainage	POD 16
PPPD	POD 6	Lower respiratory tract infection	Oral antibiotics	POD 14
PPPD	POD 5	Infected post-operative collections	IV antibiotics and radiological drainage. Returned to HDU for supportive care	POD 52
DP	POD 4	Lower respiratory tract infection	IV antibiotics. Prolonged HDU stay	POD 28
Excision of choledochal cyst	POD 2	Tachycardia and hypotensive with a raised WCC	Supportive measures. No evidence of sepsis	POD 12
Right hemi-hepatectomy	POD 6	Liver abscess	IV antibiotics. Radiological drain inserted.	POD 18
DP	POD 6	Lower respiratory tract infection	IV antibiotics.	POD 15
PPPD	POD 4	Infected post-operative collection	IV antibiotics. Radiological drain inserted.	POD 20
PPPD	POD 12	Bibasal lower respiratory tract infection	IV antibiotics.	POD 22
Right hemi-hepatectomy	POD 10	Lower respiratory tract infection	IV antibiotics.	POD 19
Mortality				
Operation	**Day SIRS/Sepsis criteria met**	**Cause**	**Outcome**	**Death**
PPPD	POD 2	Adult respiratory distress syndrome (ARDS) secondary to pancreatic leak	IV antibiotics and ventilator and dialysis support. Re-look laparotomy on POD 3.	POD 4
PPPD	POD 1	Massive pancreatic leak leading to entire small bowel ischaemia.	Re-look laparotomy. Degree of damage to small bowel unsalvageable.	POD 3

Figure 1

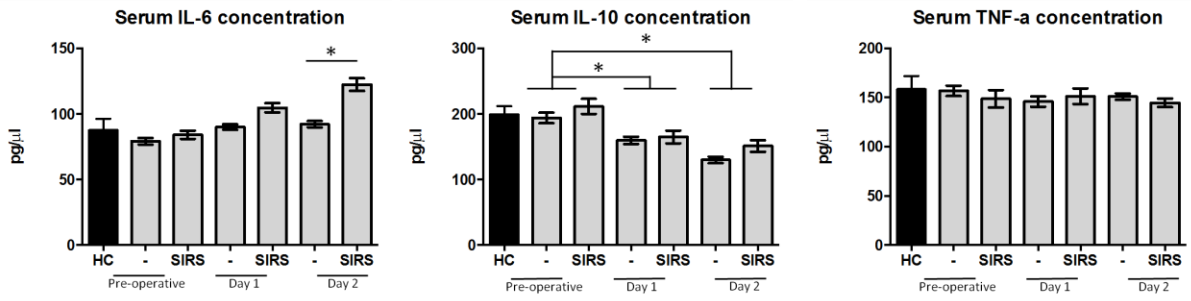


Figure 1. Serum concentrations of IL-6, IL-10 and TNF-α production pre-operatively, on day 1 day 2 in patients who have an uneventful recovery (-) and patients who develop post-operative SIRS (SIRS). *p<0.05

Figure 2

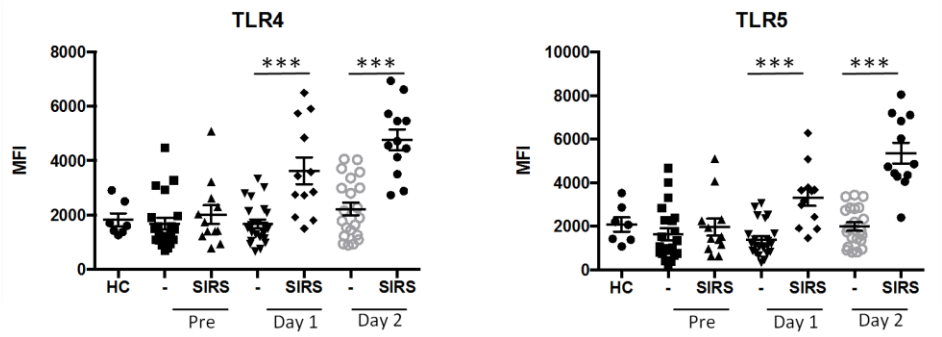


Figure 2. Expression of pre-operative and early post-operative (day 1 and day 2) TLR4 and TLR5 expression on total CD14+ monocytes in patients who develop SIRS (SIRS) and those who have no complications (-). TLR4 and TLR5 expression in healthy controls (HC) is also shown.

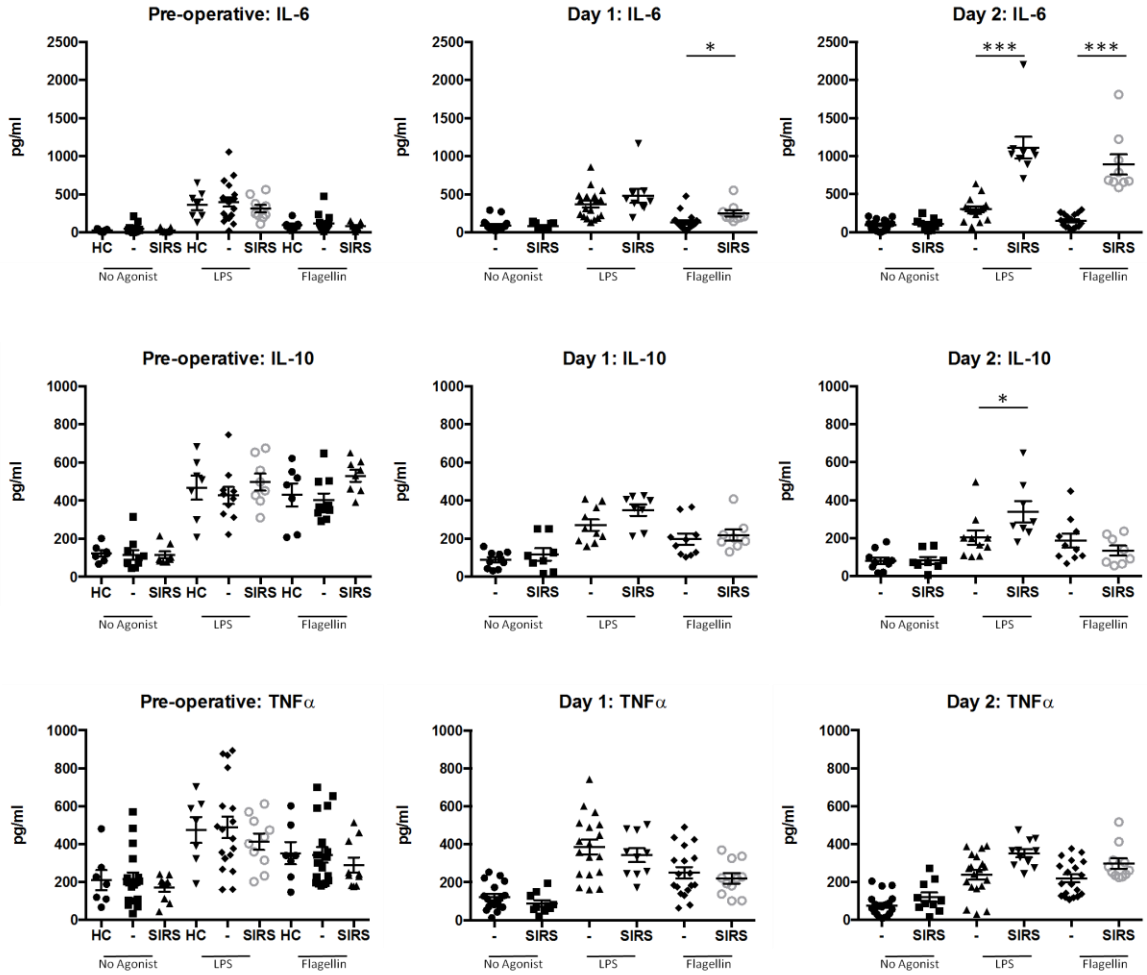


Figure 3. Unstimulated, LPS- and flagellin stimulated IL-6 , IL-10 and TNF production pre-operatively and on post-operative days 1 and 2 following major HPB surgery. Cytokine production was compared between patients who did not develop complications (-) and patients who develop SIRS (SIRS).

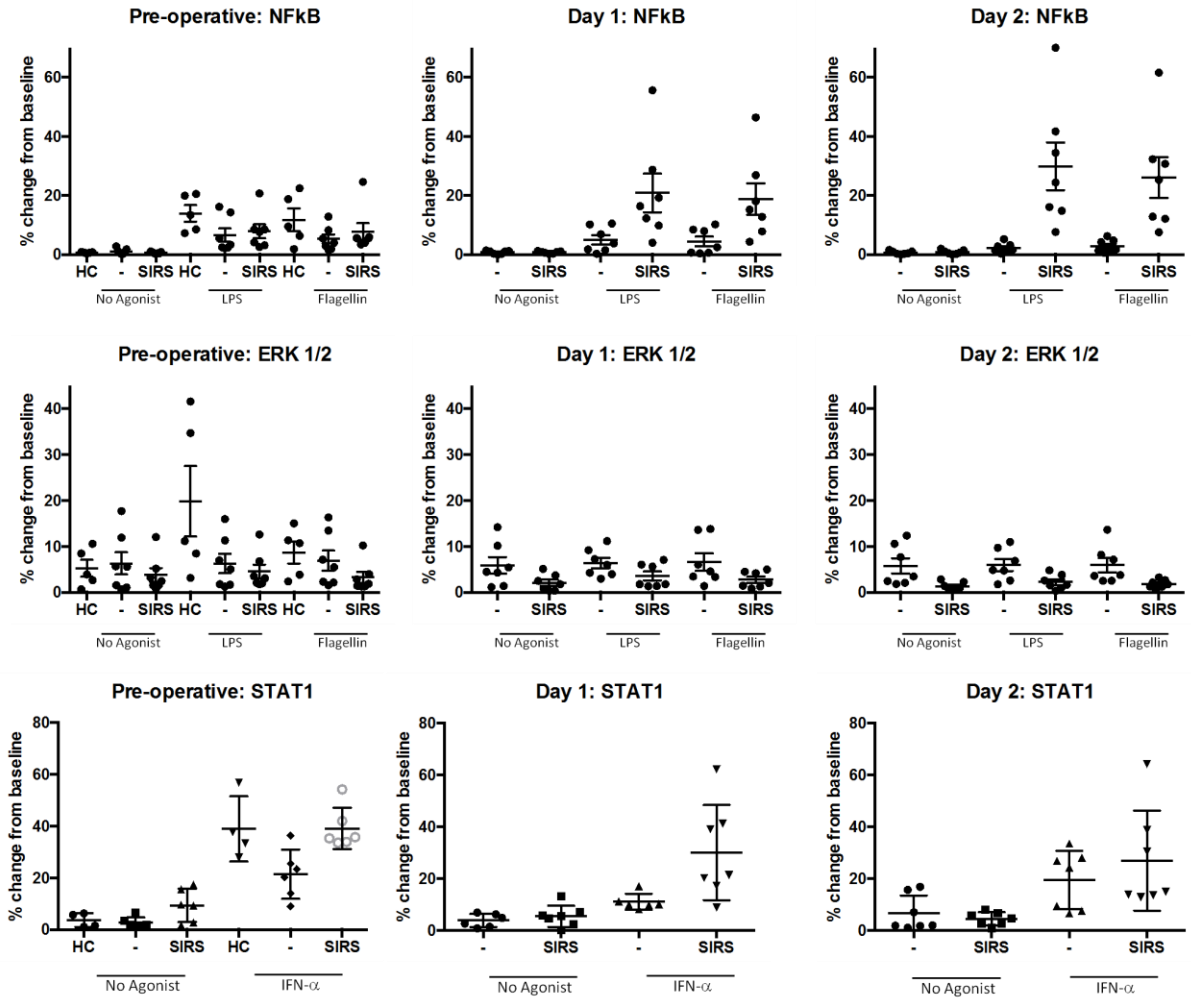


Figure 4. Unstimulated, LPS-stimulated and flagellin-stimulated NF-κB and ERK ½ phosphorylation before surgery (pre-op) and on day one (Day 1) and day two (Day 2) following surgery. Changes in unstimulated, LPS, flagellin-stimulated monocyte ERK1/2 phosphorylation before surgery (Pre-op) and on day one (Day 1) and day two (Day 2) following surgery in patients who have an uneventful recovery (-) and those who develop SIRS (SIRS)**.** Changes in unstimulated and IFNa-stimulated monocyte STAT1 phosphorylation before surgery (Pre-op) and on day one (Day 1) and day two (Day 2) following surgery.

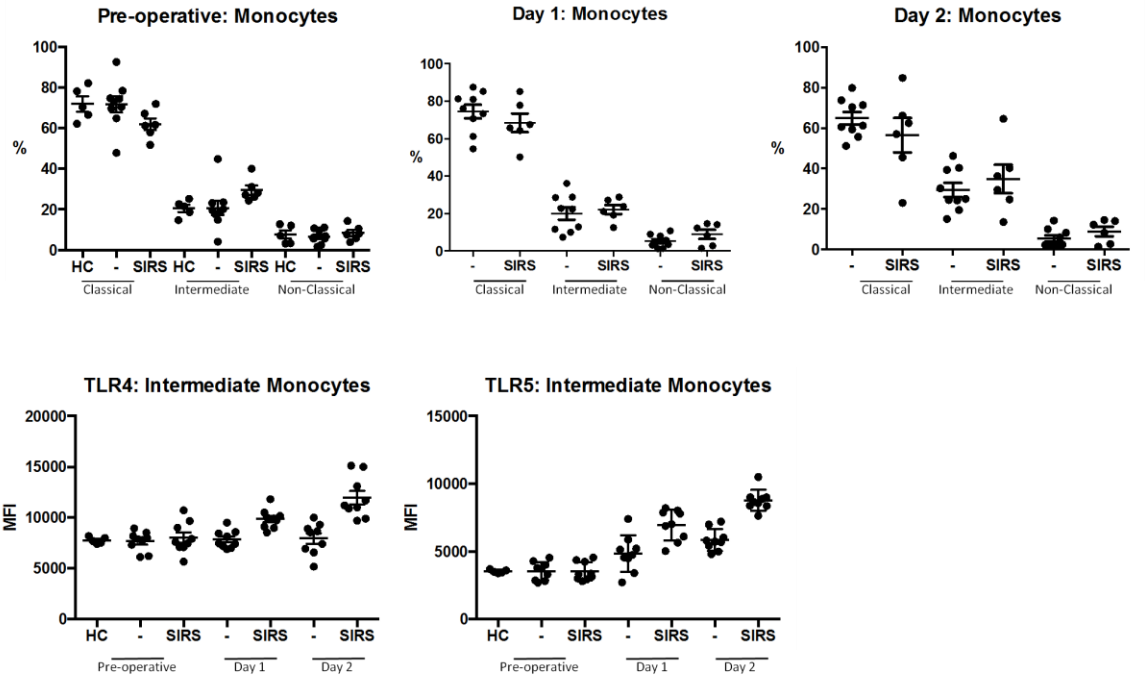


Figure 5. Monocyte subpopulations in the peri-operative period. The percentage change in classical (CD14++/CD16-), intermediate (CD14++/CD16+) and non-classical (CD14+/CD16++) monocytes between patients who have an uneventful recovery (-) and those who develop SIRS (SIRS) is shown.
Expression of TLR4 and TLR5 expression on intermediate CD14++Cd16+ monocytes from healthy controls (HC) and from patients who develop SIRS (SIRS) and those who have no complications (-).

Figure 6

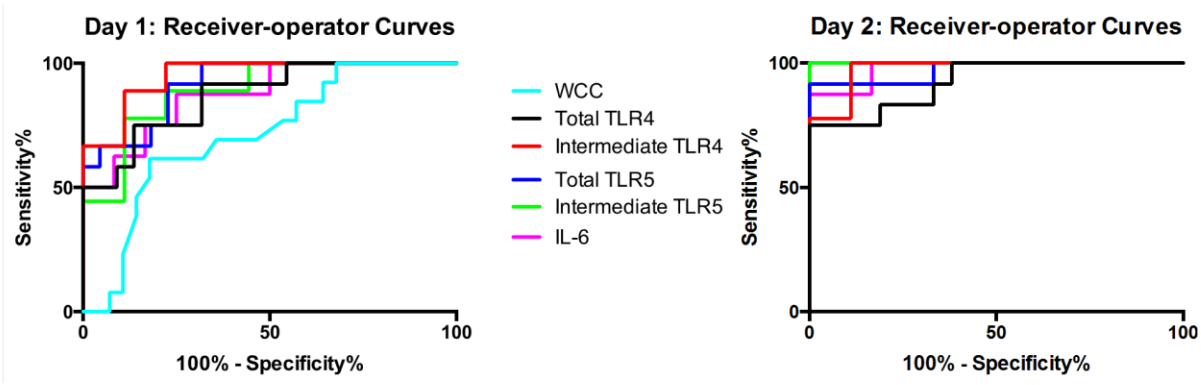


Figure 6. Accuracy of predicting SIRS. Receiver-operator curves showing accuracy of data from day 1 and day 2 in predicting the development of postoperative SIRS. wcc=total white cell count (from clinical laboratory). Total TLR = median fluorescence intensity of TLR on CD14+ cells. Intermediate TLR = median fluorescence intensity of TLR on CD14+CD16+ intermediate monocytes. IL-6 = serum concentration measured by ELISA.

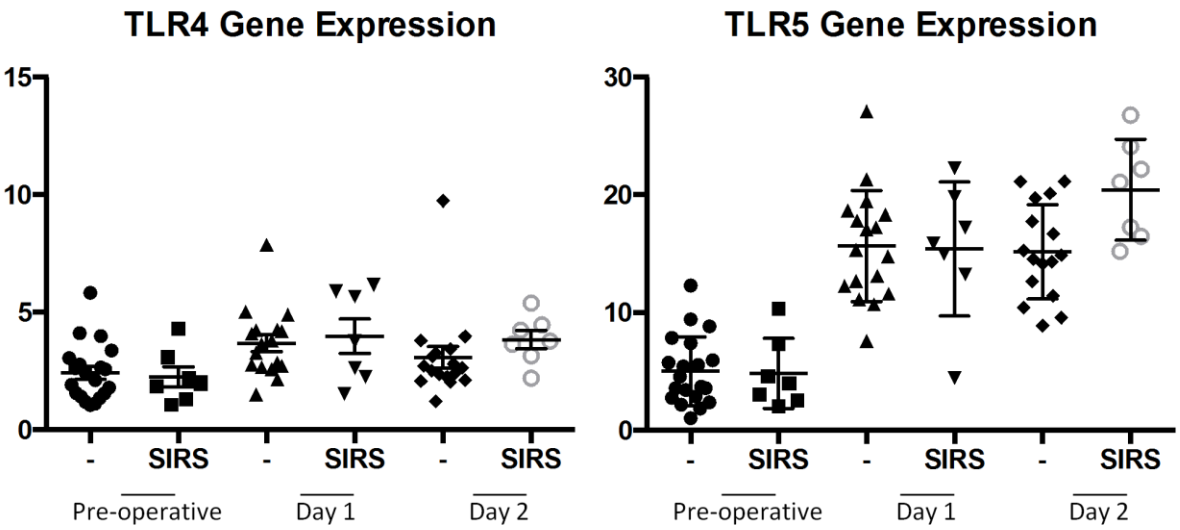
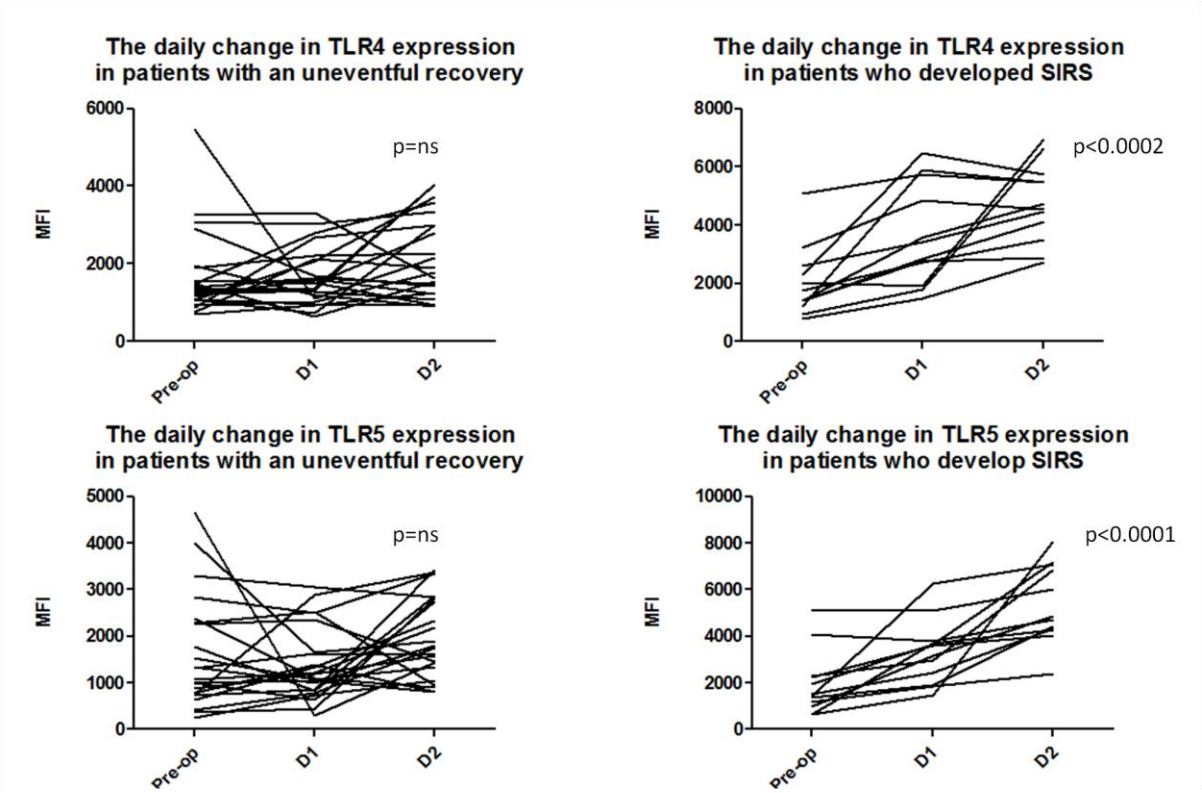
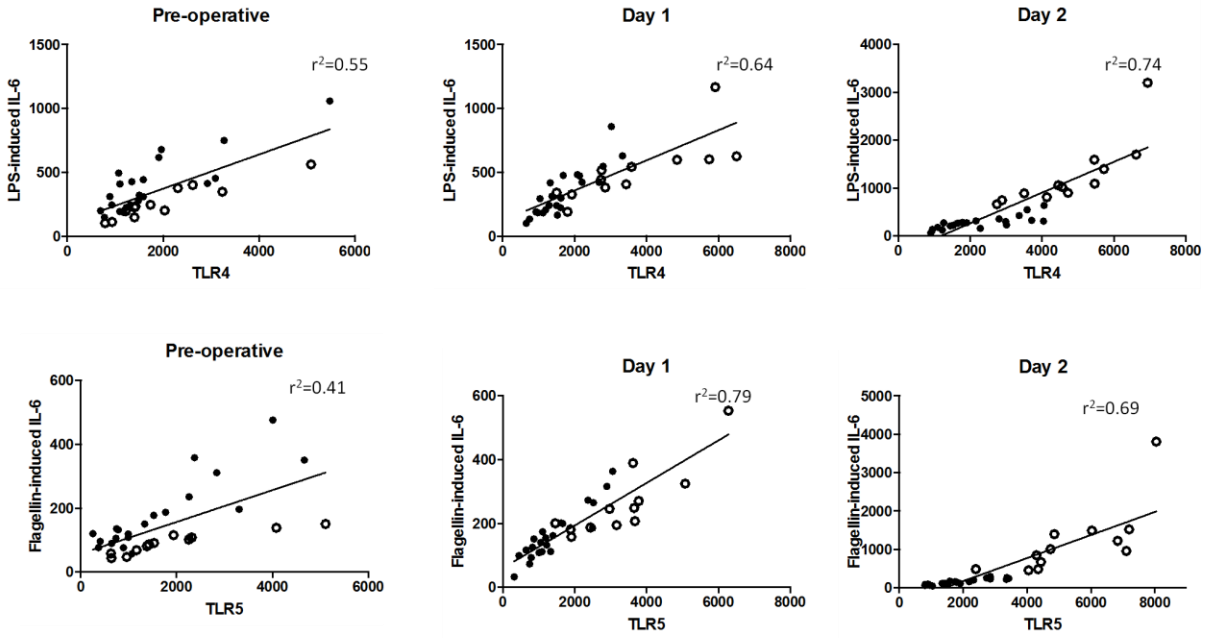


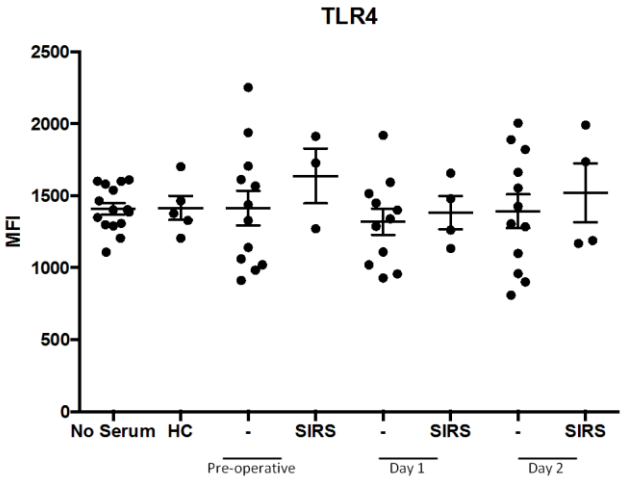
Figure 7. TLR4 and TLR5 gene expression in Cohort B

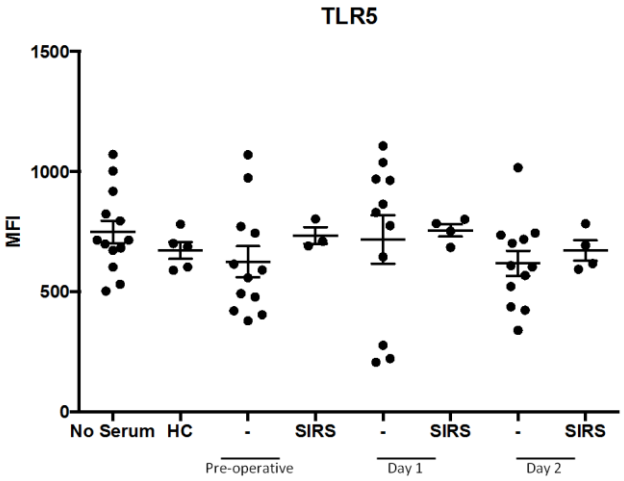


Supplementary Figure 1



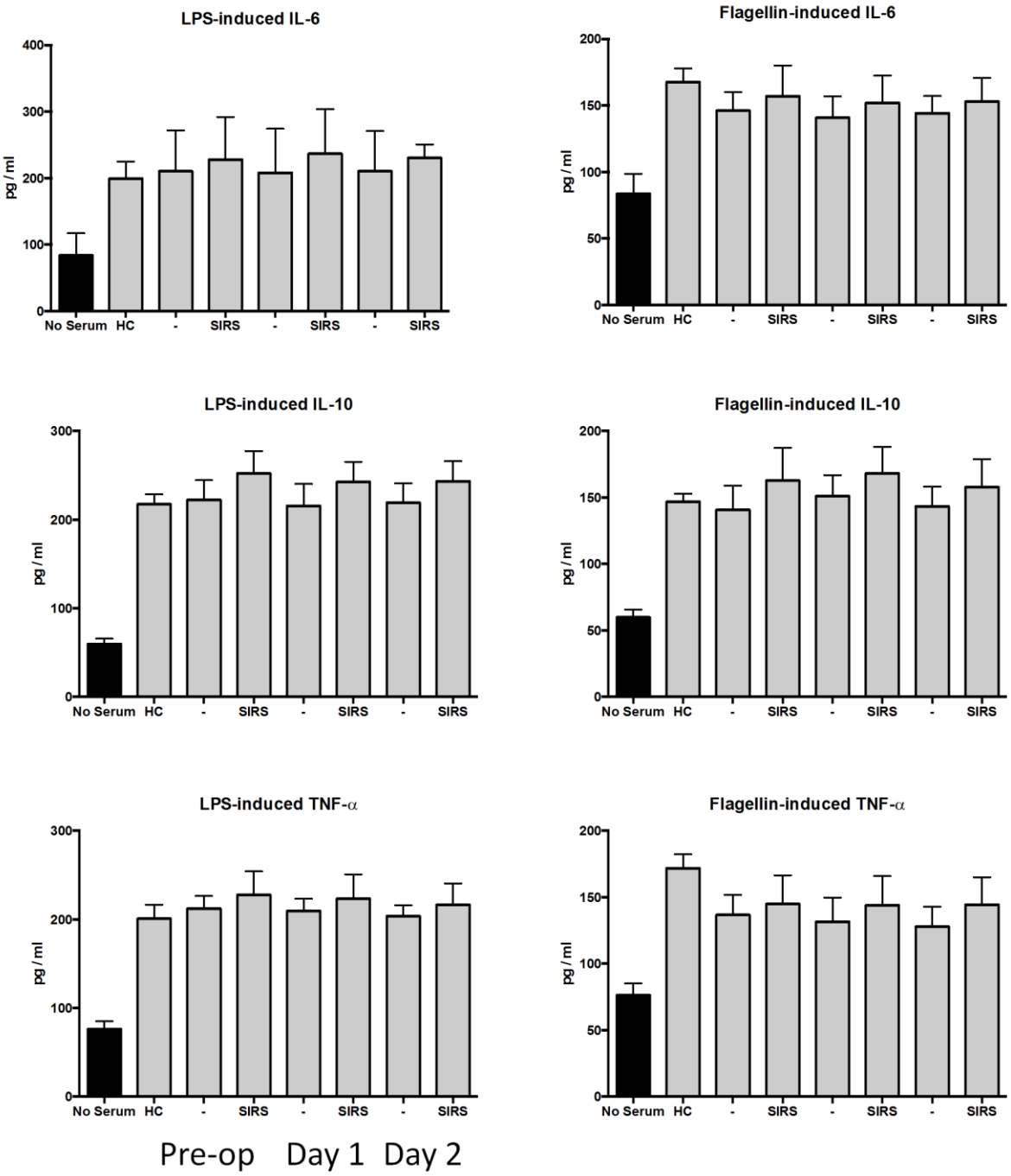
Supplementary Figure 2. Correlation between the pre-operative, day 1 and day 2 expression of TLR4 and LPS-stimulated IL-6 production (a-c) or TLR5 and flagellin-stimulated IL-6 production (d-f) in patients who have an uneventful recovery (closed circles) and those who develop SIRS (open circles). The r^2 coefficient is presented with each graph – need to add. P<0.0001 for all correlations.



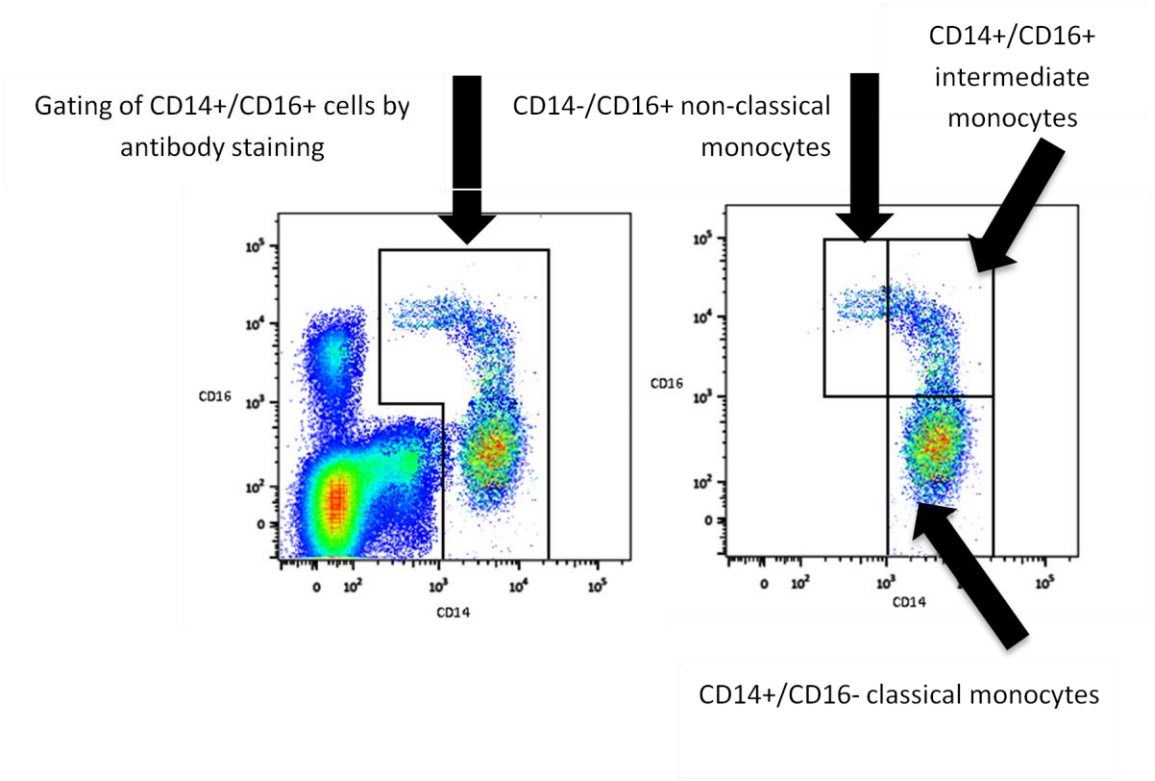


Supplementary Figure 3. TLR4 and TLR5 expression on PBMCs from healthy volunteers with and without incubation with patient sera. TLR expression was evaluated on pooled PBMCs without added serum (No Serum), maintained in media conditioned with serum from healthy controls (HC), patients who had an uneventful recovery (-) or patients who developed SIRS (SIRS).

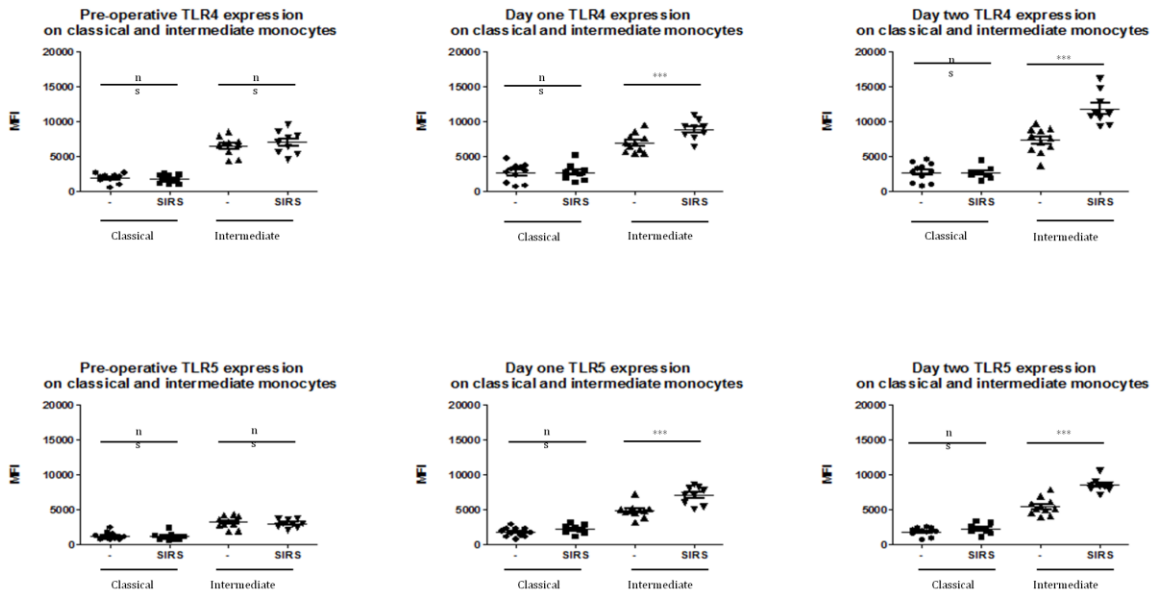
Supplementary Figure 3



Supplementary Figure 4. IL-6, IL-10 and TNF production by ELISA in supernatants from PBMCs from healthy volunteers with and without incubation with patient sera. Cytokine production expression was evaluated following stimulation with LPS or flagellin of PBMCs without added serum (No Serum), maintained in media conditioned with serum from healthy controls (HC), patients who had an uneventful recovery (-) or patients who developed SIRS (SIRS).



Supplementary Figure 5



10.3 Patient information sheet and consent form

PATIENT OR PARENT/CARER'S INVITATION TO PARTICIPATE IN A RESEARCH STUDY:

INFLAMMATION AND IMMUNITY IN DISEASES INVOLVING THE DIGESTIVE SYSTEM IN CHILDREN AND ADULTS

Digestive Diseases Clinical Academic Unit

Barts and the London, Queen Mary's School of Medicine and Dentistry

& Barts and the London NHS Trust

We invite you / your child (DELETE AS APPLICABLE) to participate in research which we think may be important. The information which follows tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. It is entirely your choice as to whether or not you do take part. Please ask any questions you want to about the research and we will try our best to answer them.

Why have I or my child been asked to participate in this study?

You are being asked to participate in this study because the the test(s) you are scheduled to have may give information about the organs of your digestive system (such as the stomach, bowel, liver and pancreas) and they have recommended that you undergo a blood test and/or another test for further investigation.

What is the Digestive Diseases Clinical Academic Unit?

The Clinical Academic Unit is a merger of hospital and university staff from the Departments of Adult and Paediatric Gastroenterology, Hepatology, Surgery, Radiology, Oncology & Pathology. We have a major role in leading research into diseases of the oesophagus, stomach, bowel, liver, pancreas and related organs in adults and children. The research we perform is in the laboratory studying individual cells and molecules relevant to the development and treatment of these diseases. We are able to do a lot of work using special cells bought from companies and grown in pots but it is vital to be able to compare these results with specimens collected from real patients with (and without) diseases.

You/your child is due to undergo tests involving collection of a blood, tissue or fluid samples and may have one of the conditions that we are studying.

What we are asking?

In the routine clinical care of you and/or your child, blood and tissue samples will be taken (or in some instances discarded eg saliva/stool/urine or tissue from an operation). In order to continue to carry out the research work outlined above, we would like your permission to take and store a little extra amount or a spare part of these samples. This sample may include blood, stool, urine, other fluids or tiny fragments of biopsies that have been taken as part of a procedure. These are detailed below and usually only one or two will apply to you/your child at a time.

Some of these specimens may be stored in a freezer for future projects, others may be used for approved studies quickly and not stored. Although you/your child may not turn out to have any disease it is very important for us to be able to compare patients with diseases to others without.

In addition, we would like to collect and store important and relevant clinical details (e.g. age, sex, medication, disease, findings from tests) for analysis with the specimens. These data will be kept in secure anonymised databases and you/your child will not be identifiable to anyone who is not providing your clinical care in the hospital.

Blood tests

If you need to have a blood test, we would like your permission to collect extra blood; up to 30 ml from adults (2 tablespoons) or 10ml from children (2 teaspoons).

Endoscopy

During examination of the bowel we will be taking 12-16 biopsies or more (small bites of the surface of the wall of the bowel, the size of a pin head) which are looked at under a microscope to help us know what you/your child's problems could be due to. We also sometimes take brushings of the gut or bile ducts or even fluid samples. This is a routine and important part of the test. In addition to the normal samples, we would like to take a maximum of 4 extra biopsies in two separate areas of the bowel, or extra brushing or fluid samples which we will use for ongoing projects within our laboratories.

Tissue or fluid sample through the skin

Only a certain amount of tissue is needed from any biopsy for routine clinical care. However we often find that in the procedure to take a tissue sample a tiny bit extra has been taken. This is not

unusual, but it does mean that if you require a biopsy to be taken (for example of the liver) through the skin and there is some spare tissue in that sample then we would like to take and use that for our studies.

If you need to have a sample of fluid taken from the abdomen or around the lungs, and if more is taken than is necessary for your routine clinical needs, we would like to take some of this extra fluid for our studies.

Surgical Procedures

During your operation, it is likely that your surgical team will take biopsies or resections of tissues for diagnostic purposes. If there is some spare tissue in that sample that would normally be discarded then we would like to take that small extra amount for our studies.

Saliva, stool & Urine

In some cases, if you agree, we would also like to collect a small (teaspoon sized) stool specimen and/or a urine specimen or a small amount of saliva.

NOTE:

1. **You will not be asked to undergo a blood test or procedure unless you need it for your routine clinical care.**

2. **You will not be exposed to any extra risk for the sake of these tissue or fluid samples.**

3. **If, after routine amounts of tissue have been taken for your care, we find that there is not enough tissue for research purposes then no further attempts to take tissue will be made solely for research.**

How will this affect me/my child?

Priority will always be given to collecting specimens needed for our planned tests. If there are any difficulties during the collection of these specimens (e.g. in collecting blood or during a procedure) then the doctors will **not** collect specimens for research.

Taking the very small amount of extra blood will not mean any significant extra risk or harm to you/your child.

Biopsies during endoscopy

Taking biopsies during an endoscopy is painless. There is a negligible risk of bleeding from biopsies. For a small number of patients who may be more prone to bleeding we perform tests to confirm if that may be the case and if so would not include them in this study, the majority do not require any extra blood tests. In the very rare reports of bleeding in the medical literature it is minor and resolves spontaneously within 1-2 days without requiring any extra treatment or stay in hospital.

Tissue or fluid sample through the skin

The reasons for collecting these samples and its risks and benefits will be explained to you by the clinical team before you agree to it. There is no added risk in donating spare samples to the research team. You will not have a biopsy unless you need it for clinical reasons and we will not perform the biopsy or fluid sample any differently because you have agreed to take part in this project.

What research is being done?

Broadly speaking we are studying how chemicals, molecules and cells in the organs of the digestive system (liver and bowel) interact with each other and other substances, cells and tissues around the body. We are particularly interested in these substances and cells in patients with the diseases we are interested in (whether on or off treatment). In addition, we are interested in these substances and cells in patients who do not have disease in order to make important comparisons.

Our studies involve measuring molecules and studying cells in normal and abnormal specimens and compare them to see if there is a difference. We also measure substances before and after starting new treatments to see how they change. Within this remit we have numerous projects and are studying many substances. We also plan to study how the genetic make-up of patients with inflammatory bowel disease (Crohn's disease or Colitis), coeliac disease or liver disease may lead to them getting the disease by influencing the reactions described above.

The research included within this application will not have any impact on the care or treatment of you/your child (including any direct benefit or harm). All our research is aimed to increase understanding of how and why diseases occur, how treatments work and how we may be able to improve treatments in the future. If any studies may affect the care of individual patients or children then a separate consent form will be produced.

Feedback

As the information found is not of any clinical relevance to you or your child we will not routinely be feeding back any information to you. However it is possible for you to know more about the research and what has happened to any specimens collected:

- You are welcome to visit the laboratories and talk to the staff there about the research that we are doing, please contact Dr Croft if interested or discuss with the person collecting the consent.
- We have a web page http://www.icms.qmul.ac.uk/centres/digestivediseases/index.html where the titles of recent publications from our departments can be found.
- Dr Croft maintains a file of ongoing projects (which may use specimens from patients) which can be seen on request.

- As specimens are not anonymised senior doctors in the department will be able to identify which project individual specimens have been used for if you or your child are interested at a later date.

Is this anonymous?

While collection of these specimens will not be anonymous only doctors or nurses involved in your or his/her clinical care will have access to your or his/her name. This allows us to go back to the hospital notes if we need to identify important and relevant information later on. Within the laboratory anonymity will be maintained by allocating a number (rather than name) to the specimens. Only your consultant or staff from the wards/clinics under his/her supervision will be able to link your name to the specimen.

What happens if I or my child are not keen or change our minds?

You and/or your child are entirely at liberty to decide not to participate or drop out at any time and this will not affect his/her care in any way. If after specimens have been collected you change your minds contact us and the specimens will be discarded if they have not already been used.

Is this ethical?

Specimens will be collected as part of the Human Tissue Authority licenced Tissue Bank at Barts & The London. We have gained approval from the East London and City Health Authority Research Ethics Committee for the collection and storage of these specimens and data from the medical notes, and each project they are used in.

What happens if we are worried or if there is an emergency?

You will always be able to contact someone to discuss your concerns and/or to get help:

Adults: Department of Gastroenterology - 020 7377 7442

In emergencies contact the endoscopy unit (0207 377 7218), or out of hours ask for the Gastroenterology Registrar via the Royal London switchboard (0207 377 7000).

Children: Department of Paediatric Gastroenterology – 020 7943 1339/1353.

In emergencies ask for the Paediatric SpR (covering gastroenterology) via the switchboard at the Royal London (0207 377 7000).

What if Something Goes Wrong?

We would not expect you to suffer any harm or injury because of your participation in this study. If you are harmed by taking part in this study, there is no special compensation arrangement. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Please contact Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please telephone 020 7377 6335, minicom 020 7943 1350, or email pals@bartsandthelondon.nhs.uk, you can also visit PALS by asking at any hospital reception.

Any other Questions?

Any other questions can be addressed by writing to:

Dr Nick Croft

c/o Digestive Disease Tissue Bank, David Hughes Building, Royal London Hospital, Barts and the London NHS Trust.

Tel: 0207 943 1339.

WRITTEN CONSENT FORM

TITLE: Inflammation and immunity in diseases involving the digestive system in children and adults
REC Number: P/01/023

Specimen(s) (please tick):	**Extra or Spare/Discarded**	**Tick those collected**
Blood	Extra (max 10 ml children, 30 ml adults)	
Blood for genetics studies	Extra (max 10 ml children, 30 ml adults)	
Endoscopic biopsy	Extra (maximum 4 in two areas)	
Surgical specimen	Spare	
Liver Biopsy	Spare	
Fluid Tap through the skin	Spare	
Urine	Discarded	
Stool	Discarded	
Saliva	Discarded	

Name of Patient/Volunteer :

Hospital/NHS Number

Initials

The study organisers have invited me/my child to take part in this research.	
I confirm that I have read and understand the information sheet version 2.1 dated 25th November 2010 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
I know what me/my child's part will be in the study and I know how long it will take. I know how the study may affect me/my child. I have been told if there are possible risks.	
I understand that me/my child should not **actively** take part in more than 1 research study at a time.	
I know that the local East London and The City Health Authority Research Ethics Committee has seen and agreed to this study	
I understand that personal information is strictly confidential: I know the only people who may see information about my part in the study are the research team or an official representative of the organisation which funded the research.	
I freely consent for me/my child to be a subject in the study. No-one has put pressure on me.	
I know that I or my child can stop taking part in the study at any time. I understand that my / my child's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	

I understand that the sample may be used for research aimed at understanding the genetic influence on diseases of the digestive system but the results of the investigations are unlikely to have any implications for me/ my child personally	
I understand that the study blood samples and any spare/discarded specimens are gifts to the research team.	
I know that if there are any problems, I can contact: Dr N Croft - 020 7943 1339	

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from Investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

CHILD'S INVITATION TO PARTICIPATE IN A RESEARCH STUDY: INFORMATION

INFLAMMATION AND IMMUNITY IN DISEASES INVOLVING THE GASTROINTESTINAL TRACT IN CHILDREN AND ADULTS

We invite you to participate in research which we think may be important. The information which follows tells you about it. Try to make sure you understand what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice. Please discuss this with your family and ask any questions you want to about the research and we will try our best to answer them.

Inflammation can lead to damage of the lining of the bowel or liver whereas immunity can protect you. We would like to understand more about immunity and inflammation in the bowel and how diseases of the bowel happen and how they might be avoided or treated. To do this we need to collect specimens from people both with and without problems with their bowel or liver.

You are going to undergo tests to find out more about your health or are planned to have an operation. The tests could include taking bloods, or looking with a camera inside your bowel or other samples taken through your skin. The test will be explained to you and your family before it is done. When we look inside your tummy/bowel with the camera we also take tiny (and painless) bites of the lining (called biopsies, the size of a pinhead) to look at these under a microscope. This will be explained to you before you undergo the test.

What we are asking

We would like to ask your permission to collect either extra specimens (one or two teaspoons of blood) and/or up to four extra biopsies in two areas of the bowel (maximum 8 biopsies) or collect spare parts of samples which are more than needed for the tests or are specimens which would normally be thrown away (such as stool ('poo') or urine ('wee'), or after an operation), which we can use for research in our laboratories.

What are we going to do with them?

We are going to use these specimens to help research into a range of diseases of the bowel that can affect both children and adults, one of which you may have (for example Crohn's, colitis, ulcers, coeliac disease, cystic fibrosis, food allergy, infections, liver diseases). Even if you do not have any of these problems it is important for us to be able to compare children with and without these diseases. Some of these specimens may be stored in a freezer others will be sent straight to the laboratory for tests.

All our research is intended to increase understanding of how and why diseases occur, how treatments work and how we may be able to improve treatments in the future.

The research involves studying how substances within the body (e.g. foods, nutrients, bugs) can send messages to the cells under the lining of the bowel and cause, prevent or heal damaged areas. We do this by measuring molecules in normal and abnormal areas and compare these to see if there is a difference. Within this we have numerous projects and are studying many substances. We also plan to study how genetic make-up may lead to children getting Crohn's or colitis or coeliac disease and whether this make-up can alter how effective the treatments are.

How will this affect me?

We will always collect specimens needed for your planned tests before any research specimens. If there are any problems during the collection of these specimens (e.g. in collecting blood or during the gastroscopy/colonoscopy) then the doctors will not collect specimens for research.

Taking extra blood will not cause you any problems. You are also extremely unlikely to have any problems if we collect extra bits of the lining of the bowel. Very rarely minor bleeding may occur however this resolves on its own within 1-2 days without requiring any extra treatment or stay in hospital.

The research itself is not relevant to your care. If in the future we would like to perform research that may be directly relevant to you we will contact you to see if you agree.

Do the specimens have my name on them ?

The people in the laboratory will not know your name as your specimens will have a number. Only Dr Croft and/or one of the other consultants (Prof Sanderson, Dr Naik, Dr Rawat, Dr Meadows) or hospital staff supervised by them will be able to know this and link it to specimens collected.

Can I find out more about the research ?

- You are welcome to visit the laboratories and talk to the staff there about the research that we are doing, please contact Dr Croft if interested or discuss with the person collecting the consent.
- We have a web page (http://www.icms.qmul.ac.uk/centres/gastroenterology/index.html) where the titles of recent publications from our department (as well as our colleagues the adult gastroenterologists) can be seen.
- Dr Croft's secretary will also have a file of all ongoing projects (which may use specimens from patients) which can be seen on request (e.g. in outpatients).
- Dr Croft will be able to identify what study your specimen was used for if you are interested at a later date and will be happy to discuss it with you and your family.

What if I don't want to be included or change my mind ?

You are entirely free to decide not to participate or drop out at any time and this will not affect your care in any way. If a specimen has been collected and you change your mind it will be destroyed at your request.

CHILD'S INVITATION TO PARTICIPATE IN A RESEARCH STUDY: CONSENT

INFLAMMATION AND IMMUNITY IN DISEASES INVOLVING THE GASTROINTESTINAL TRACT IN CHILDREN AND ADULTS

- ***I HAVE READ AND KEPT THE INFORMATION SHEET…***
- ***I UNDERSTAND THE RESEARCH WILL NOT AFFECT MY CARE OR TREATMENT...***
- ***I HAVE SPOKEN TO THE DOCTOR AND MY FAMILY AND ASKED ANY QUESTIONS I WOULD LIKE…***
- ***I REALISE I DO NOT HAVE TO DO THIS …***
- ***AND CAN STOP OR WITHDRAW AT ANY STAGE WITHOUT AFFECTING MY TREATMENT….***
- ***I AM HAPPY TO GO AHEAD.***

Signed ***……***

Name ***……***

Witnessed ***……***

Signature ***……***

Date

Appendix:

Outline of Current or Proposed Projects within the Adult and Paediatric Gastroenterology Clinical Academic Unit:

There follows annotated outlines of some of the projects on-going or planned within the departments of Adult and Paediatric Gastroenterology. Some aspects of these (e.g. in 1, 3, 7 and 8) have also been through the full ethical process.

1. ***Epithelial and lamina propria cell signalling in the mucosa of subjects with inflammatory bowel disease:***

Professor Ian Sanderson - Professor of Paediatric Gastroenterology

Professor D Rampton – Professor of Clinical Gastroenterology

Dr Rachel Levi - Clinical Lecturer in Paediatric Gastroenterology

Dr Jenny Epstein – Research Fellow in Paediatric Gastroenterology (CICRA)

Dr Khaled Khatab – Research Fellow

Dr Giovanni Monteleone - Research Fellow (taken over from Dr Catriona MacKenzie previous application **P/97/330)**

By measuring proteins, contained within cells of the gastrointestinal mucosa, which are fundamental to the control of production of pro- and anti-inflammatory cytokines it is possible to understand more about the molecular mechanisms involved in the development of intestinal inflammation in IBD. Up or down regulation of these proteins (both in active disease or after treatment or after exposure to other compounds for example nicotine) can lead to enhanced cytokine production and tissue damage (or conversely recovery). Signalling molecules currently being studied include TOLL 4/2, SMAD 4, SMAD 7 and a range of cytokines (including IL-1, Il-8, IL-12, IL-18, TGF-beta).

2. ***Apoptosis of epithelial cells in IBD and intestinal graft versus host disease***

Dr Nick Croft - Senior Lecturer in Paediatric Gastroenterology

Pro-inflammatory cytokines (e.g. IL-1, IL-8, IL-6, TNF) are known to induce apoptotic cell death of intestinal epithelial cells. This in turn leads to loss of the epithelial cell barrier and subsequent inflammation and tissue damage. Apoptosis is also a key histological (and diagnostic) feature of biopsies from children with intestinal graft versus host disease. Whether it is also a feature of chemotherapy induced gut damage has not been established. Apoptotic cell death is induced by a group of enzymes within the cells called caspases. The first aim of this work is to study the mechanisms in which the caspases are stimulated by cytokines to cause cell death and tissue damage

in diseases of the gastrointestinal tract such as IBD and graft versus host disease. The secondary aim is to attempt ameliorating the process (and thus reducing tissue damage) using caspase inhibitors.

3. Nutrition and Growth Factors in Inflammatory Bowel Disease (application P/00/110)

Dr N Croft - Senior Lecturer in Paediatric Gastroenterology

Prof I Sanderson – Professor of Paediatric Gastroenterology

Dr Lucinda Hall – Reader in Medical Microbiology

Growth failure is a common problem in children with IBD. Insulin like growth factor 1 (IGF-1) is a major stimulant of growth in children. In vitro the release of IGF-1 can be reduced by pro-inflammatory cytokines (IL-6 and TNF-alpha). These cytokines are raised in active IBD. We have recently shown that treatment with enteral nutrition can reduce inflammation and increase IGF-1 in children with IBD. By measuring serum and tissue levels of cytokines and growth factors plus the response of bacteria and bacterial products during active disease and during/after recovery we propose to study in more detail the causes of growth failure in IBD and the mechanisms of responding to enteral feeds.

4. Inflammation of the gut in cystic fibrosis

Dr Nick Croft - Senior Lecturer in Paediatric Gastroenterology

Dr Siobhan Carr - Consultant Paediatrician (looking after children with cystic fibrosis)

Dr Paola Domizio - Senior Lecturer in Histopathology

Recent work in children with cystic fibrosis suggests that the gastrointestinal tract can show subtle signs of inflammation (Smyth/Croft 2000). This is of great importance in understanding the disease as it is known that the gut has abnormal expression of the CFTR (cystic fibrosis transmembrane regulator) as is found in the lungs. The question remains (in the lung) whether the abnormal CFTR leads to inflammation directly or as a consequence of super-added infection. We hope to answer this by studying the gastrointestinal mucosa (which does not have pathogenic bacteria present). In this study we will plan to collect biopsies from patients with cystic fibrosis for evidence of intestinal inflammation. Histological examination will be performed by Dr Domizio and a scoring system for inflammation will be utilised. Snap frozen biopsies will then be examined for up-regulation of the mRNA and protein of suspect cytokines including IL-1, IL-8, IL-6, TNF using semi-quantitative techniques.

5. Reactive oxygen metabolites and their scavengers in the mucosa of patients with gastroeintestinal diseases (e.g. coeliac disease, food allergy and inflammatory bowel disease)

Dr Nick Croft Senior Lecturer in Paediatric Gastroenteorology

Dr David Rampton Reader in Gastroenterology

Dr James Lindsay

Prof Andy Silver

Reactive oxygen metabolites (ROM's) including superoxide and hydrogen peroxide are important mediators in inflammatory processes such as inflammatory bowel disease. The effect of antioxidant treatments is uncertain although some evidence suggests they can be beneficial. A large increase in ROM production has been shown from the colonic mucosa of subjects with active inflammatory bowel disease. No work has studied ROM production from the small intestine.

The aims of this study are to examine ROM production from the cells of the diseased small intestine including Crohn's disease, the enteropathy of coliac disease and subjects with food allergy. We also plan to examine the ROM production of blood monocytes exposed to the relevant proteins in vitro (e.g. gliadin/peanut/milk etc.), from subjects with Coeliac Disease or food allergy.

In patients with inflammatory bowel disease (IBD) (Crohn's or Ulcerative colitis) another aim of this work is to understand the potential role of 5-amino-salicylic acids, extensively used treatments for IBD, on the reactive oxygen metabolites and their scavengers (substances in the body that 'mop up' the ROM's). We plan to examine the effect of these medicines on these scavengers and the inflammation in patients by measuring a range of molecules in specimens collected during routine colonoscopies. A benefit of these drugs is thought to be a reduction in the long term risk of cancer in the large bowel. This work is intended to increase understanding of how that may occur.

6. SOCS proteins and signalling in intestinal mucosa

Dr Ping Wang Senior Lecturer in Mucosal Immunology

The unique function of the mucosal immune system is its ability to maintain the intestinal mucosa in a quiescent state of inflammation. The mechanism underlying this delicate balance is still unknown but involves the presentation of antigens from the gastrointestinal lumen via signalling pathways to the production of pro- and anti-inflammatory cytokines. We will use normal and inflamed biopsies from human intestine to assess the antigen presenting pathways (by measuring MHC class I and II and CD1 proteins) as well as intracellular signalling pathways (Suppressor of Cytokine Signalling (SOCS) Protein family). The specimens will be used for quantitative analysis by immunohistochemistry and in site hybridization. Some of the materials will be used for in vitro culture with different cytokines (IL-1, 4, 8, 10, 12, 18, IFN, TNF) and antigenic peptides.

7. The role of meprin in inflammatory bowel disease (previous application P/00/019)

Dr Nick Croft

Professor D Rampton – Professor of Clinical Gastroenterology

Matrix metalloproteases (MMP's) and their tissue inhibitors (TIMP's) control the degradation of the matrix proteins (e.g. collagen) supporting the cells of the mucosa. Damage by MMP's lead to tissue damage and ulceration. Meprin is a metalloprotease of the so-called astacin family that is expressed in the human small and large intestine. The biological function of meprin in the gut is as yet not well known. In colorectal cancer, meprin accumulates in the stroma, thereby contributing to an increased proteolytic potential during invasion of cancer cells (D. Lottaz et al. (1999) Cancer Research 59, pp. 1127-33). Aims: To investigate the regulation and function of meprin in mucosal inflammation, we will use organ-cultured biopsy specimens from patients with inflammatory bowel disease and normal controls.

8. **An investigation of the genetic determinants of susceptibility and disease behaviour in Inflammatory Bowel Disease and Coeliac disease. (specific studies approved for celiac disease- P/03/229, and 05/Q1605/89 (Oxford REC B Multisite))**

Dr D van Heel, Professor of GI Genetics

Dr N Croft

Two papers very recently published in Nature (2001) have identified for the first time a single gene (NOD2) in patients with Crohn's disease. This gene is known to produce a protein of major importance in the immune response. We propose to further this work by studying other genes and their abnormalities which via routes identified in the sections above (e.g. section 1, 2, 6 and 7) may influence both the initiation and progression of the disease. There is no clinical relevance to these findings in individual patients with IBD or coeliac disease however there will be a need to study parents and siblings (both affected and unaffected) and this will be part of a separate application to the ethical committee.

9. **Composition and metabolism of intestinal flora in Inflammatory Bowel Disease**

Dr N Croft - Senior Lecturer in Paediatric Gastroenterology

Dr Lucinda Hall – Reader in Medical Microbiology

Prof I Sanderson – Professor of Paediatric Gastroenterology

Collaborating with Sanger Institute Cambridge, Manchester University

Intestinal bacteria are known to be a vital component of inflammatory bowel diseases however there is little data examining their responses in active and then inactive disease.

The aims of this work are to examine the metabolomics and metagenomic responses of intestinal bacteria from subjects with active and inactive inflammatory bowel disease (Crohn's disease and UC). By attempting to identify alteration of specific bacterial colonies before and after treatment of their IBD (Crohn's disease) we plan to identify specific responses and understand more about the role of bacteria in promoting or reducing inflammation.

10. **Is the protein filaggrin expressed in the human gastro-intestinal tract?**

Dr N Croft

Prof S Mukhopadhya, Dr A Butt, Dr V Leclizio, Dr Tracey Coelho Brighton Medical School

Dr S Bovell, Glasgow Caledonian University

Background: Filaggrin is a key barrier protein produced by cells called keratinocytes in the skin. This protein aggregates the keratin cytoskeleton in the skin leading to cell compaction and formation of a chemically impermeable barrier which helps to retain water and resist the entry of allergens and toxins from the environment. Disruption of this barrier due to reduction or complete absence of filaggrin expression can lead to a chronic transcutaneous allergen entry.

In humans, research has demonstrated the presence of the filaggrin protein within the stratum corneum in the epithelium of the skin (5) and the oral cavity (6). Although filaggrin has been identified in the rodent stomach (8), presence of filaggrin in other parts of the gastrointestinal tract has not been explored. In humans, so far there have been no studies to explore the presence of filaggrin on the gut mucosa.

This study is intended to look for the presence or absence of filaggrin in control and diseased GI mucosa and its importance in the development of clinical diseases.

11. Clonal analysis of pre- and malignant gastrointestinal epithelia.

Dr S McDonald

Professor M Alison

Professor Sir N Wright

The mutation and selection theory of cancer development states that a single (stem) cell acquires a series of DNA mutations that are passed on to its progeny, which eventually leads to the formation of a cancer. If this is true within the human gastrointestinal tract, then we should be able to trace DNA abnormalities through each stage of the pathway leading to adenocarcinoma in individual glands throughout a single lesion. Previous work by our group has demonstrated that while the human stomach can show massive clonal expansion of a single mutated gland, only a small number of patients have identifiable mutations in tumour suppressor genes or oncogenes, suggesting there are other mechanisms as yet undiscovered. Furthermore, in Barrett's oesophagus we find that multiple clones arise within lesions suggesting that not only is there more than one source of potentially tumouringenic clones, but they may interact with each other to promote cancer development. Therefore, there is a great need to further examine the adenocarcinoma pathways in these tissues to identify novel clonal markers, investigate how they spread through the gastrointestinal epithelium and examine how interact with each other. We intend to investigate mechanisms of spread of genetic abnormalities through the human stomach, oesophagus and colon. To this end we would require frozen and formalin-fixed, paraffin-embedded specimens from resection operations.

12. Cell Signalling in Inflammatory Liver Diseases

Dr W. Alazawi

Professor Graham R. Foster

Inflammatory diseases of the liver are frequently caused by factors such as alcohol, virus and fat (related to diabetes). Our group is working on the mechanisms by which the liver becomes inflamed following exposure to these injuries. We are particularly interested in the role of pathogen-sensing toll-like receptor (TLR) signalling and the outcome of inflammatory responses to the activation of TLRs and other systems connected to them. Given the central role for TLR-driven inflammation in the pathogenesis of inflammatory liver diseases, this project can teach us much about the biology of inflammation as well as to identify potential new therapeutic targets for the treatment of these conditions.

13. Immunological parameters determining disease progression in hepatitis B virus

Patrick Kennedy, Senior Lecturer in Hepatology.
Graham Foster, Professor of Hepatology.
Upkar Gill, Academic SpR in Hepatology.

The quantitative and qualitative features of the inflammatory infiltrate in chronic hepatitis B virus are believed to influence disease outcome. To date, the make-up of this inflammatory infiltrate remains poorly defined. Furthermore, the influence of age on priming the immune response and shaping the avidity of the intrahepatic infiltrate has not been studied.
The aim of this work is to elucidate the factors which control the inflammatory response in hepatitis B virus. We will focus on the HBV-specific immune response and how this may be modulated by the level of virus, the expression of peptide/MHC complexes on individual hepatocytes and the role of the inflammatory milieu in shaping disease control. We will also study the non-antigen specific response and its contribution to liver injury. Having studied these two distinct pathways of liver damage, we will then focus on the influence of age and how the robust immune response reported in young people (or young adults) can contribute to more severe or marked liver disease.